의학박사 학위 논문

The roles of Gasdermin C and Cathepsin G in UV-induced skin damages

자외선에 의한 피부 손상에서의 Gasdermin C 와 Cathepsin G 의 역할

2019 년 2 월

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지도 교수 정진호

이 논문을 의학박사 학위논문으로 제출함

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The roles of Gasdermin C and Cathepsin G in UV-induced skin damages

by

Novi Kusumaningrum

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in fulfillment of the requirements for
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ABSTRACT

Gasdermin C (GSDMC) is known to be a member of Gasdermin family and expressed in the epithelial cells of various tissues including the skin. Gasdermin family members were detected in the epithelium of various tissue types in a highly tissue-specific manner and suggested to have differentiation-status specific roles. However, the function of GSDMC in the skin remains poorly understood.

Cathepsin G (CTSG) belongs to the serine protease family, and its upregulation is involved in wrinkle formation by chronic ultraviolet (UV) irradiation in the mouse skin. β-keto-phosphonic acid (KPA) is a CTSG inhibitor that shows the strongest selectivity and potency against CTSG and prevents wrinkle formation by chronic UV irradiation. However, the effect of CTSG on the basement membrane damages in the skin remains unclear.

UV radiation plays important roles in various skin damages including inflammation, sunburn, premature aging, and carcinogenesis and also induces basement membrane damages. These effects of UV are mediated by a number of changes at molecular or cellular levels such as cell cycle control, apoptosis, signal transduction, and gene expressions. However, the roles of GSDMC and CTSG in UV-induced skin damages have not been thoroughly studied.

In Chapter I, I investigated the role of GSDMC in UV-induced matrix metalloproteinases (MMPs) such as MMP-1, MMP-3, and MMP-9 expressions in human skin keratinocytes. I found that GSDMC expression is increased by UV irradiation in human skin keratinocytes. Further studies showed that GSDMC expression is increased at relatively late time points after UV irradiation and that this GSDMC induction plays important roles in the expressions of MMP-1, but not of MMP-3 and MMP-9, and the activations of ERK and JNK induced by UV radiation. In addition, I found that overexpression of GSDMC increases the MMP-1 expression and the activities of ERK and JNK and that GSDMC-induced MMP-1 expression is suppressed by inhibition of ERK or JNK activities.

In Chapter II, I investigated the role of transient receptor potential cation channel subfamily V member 1 (TRPV1) in UV-induced GSDMC expression and the signaling pathway involved in the process in human skin keratinocytes. I found that suppression of TRPV1 activity significantly reduced UV-induced GSDMC expression and activation of TRPV1 increased GSDMC expression. Next, I found that extracellular calcium and calcineurin activities were necessary for UV-induced GSDMC expression. Furthermore, I also found that UV-induced GSDMC expression was decreased and increased by knockdown and overexpression of nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1), respectively.

In Chapter III, I investigated the role of CTSG on basement membrane damages in chronically UV-irradiated hairless mouse skin. I found that KPA prevents UV-induced decrease in basement membrane components, such as type VII collagen, laminin 332 and perlecan, in the basement membrane zone (BMZ), and UV-induced breakage of lamina densa and UV-induced shortening of hemidesmosomes. KPA prevents UV-induced CTSG and MMP-13 expressions in chronically UV-irradiated hairless mice. Increase in neutrophil infiltration by UV irradiation is prevented by KPA treatment. UV-induced wrinkle formation is also prevented by topical KPA treatment.

In Chapter IV, I performed the screening of CTSG inhibitor using plant extracts and drugs to discover other substances that are safe for clinical application in human skin instead of KPA. I found that Jeju indicum and triamcinolone acetonide have the highest inhibition activity against CTSG among other plant extracts and drugs, respectively. However, the use of drugs for clinical application in human skin should consider its side effects, especially in the long-term application.

Briefly, I suggested that the increase in GSDMC by UV radiation contributes to UV-induced MMP-1 expression through the activation of ERK and JNK pathways. I found that TRPV1 plays an important role in the induction of GSDMC expression by UV radiation and that UV-induced GSDMC expression is mediated via the calcium/calcineurin/NFATc1 pathway. I also showed the involvement of CTSG in UV-induced basement membrane damages in the skin, and inhibition of CTSG may be a useful strategy for the prevention of UV-induced basement membrane damages and

photoaging. I discovered the substances that can inhibit CTSG activity and are safe for clinical application in the human skin instead of KPA.

Keywords: Gasdermin C, Cathepsin G, Matrix metalloproteinases, Ultraviolet, skin damages, gene expressions, signaling pathway

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INTRODUCTION

Ultraviolet (UV) light

UV light is one of the major environmental factors that affect human health. The skin is the largest organ of the body, through which humans interact with their environment. Consequently, the skin is frequently exposed to UV radiation. The effects of chronic UV exposure on the human skin are manifested as cutaneous photoaging [1-4]. Repeated UV exposure induces a variety of skin damages, which are characterized by different clinical changes, such as skin carcinogenesis, premature skin aging, formation of deep wrinkles, roughness, pigmentation disorders, and decrease in skin elasticity, and histological changes including loss of polarity, cellular atypia and damage to the basement membrane structures [5-9]. The effects of UV may be mediated by various types of cellular-level changes, including autophagy, cell cycle control and cell death, as well as molecular-level changes, including signal transduction pathways and gene expressions [10-12]. UV irradiation may also play a role as a broad activator of cell surface growth factor and cytokine receptors [10, 11]. Activation of ligand-independent receptors stimulates various downstream signaling pathways that control the expression of a number of genes [10-12].

Gasdermin C (GSDMC)

The Gasdermin (GSDM) superfamily consists of Gasdermin family genes (GSDMA, GSDMB, GSDMC, and GSDMD in humans) and Gasdermin-related genes (DFNA5 and DFNB59 in humans) [13]. GSDM family members were differentially expressed in the epithelial cells of various tissue types, including the skin, esophagus, stomach, trachea, and spleen in a highly tissue-specific manner, and suggested to have differentiation-status specific roles [14-16]. GSDMC was known as melanoma-derived leucine zipper-containing extranuclear factor (MLZE) [16, 17]. Several studies have suggested that GSDMC may be involved in the course of tumorigenesis such as acquisition of

metastatic potential in melanoma cells or promotion of cell proliferation in colorectal carcinogenesis and that GSDMC may function as an oncogene [17, 18]. Even though the expression of GSDMC in the skin has been demonstrated, its function in the skin remains unclear.

Cathepsin G (CTSG)

CTSG, a single-chain 28.5-kDa glycoprotein, is one of the three serine proteases of the chymotrypsin family, the other two members being neutrophil elastase (NE) and proteinase 3 (PR3). It was shown to be expressed in the azurophil granules of neutrophilic polymorphonuclear leukocytes [19-21]. Previous reports have shown that chronic UV exposure leads to the induction of CTSG, indicating that CTSG is a potential biomarker of human skin photoaging [20, 22]. The expression or activity of CTSG can be inhibited by β-keto-phosphonic acid (KPA) [22-24]. KPA is a non-peptide type of CTSG inhibitor that shows the most specific inhibitory activity against CTSG [24, 25]. It has been reported that the inhibition of CTSG by KPA contributes to the prevention of UV-induced photoaging via inhibition of fibronectin fragmentation and MMP upregulation [23, 24]. Even though many reports have demonstrated the role of CTSG in skin photoaging, the effect of CTSG on the basement membrane damages in the skin remains unknown.

Matrix metalloproteinases (MMPs)

MMPs comprise the family of zinc-dependent endopeptidases that are known to be important factors for degradation of extracellular matrix proteins [26-28]. MMP family members can be categorized into four different subfamilies: collagenases, stromelysins, gelatinases, and membrane-type MMPs [28-30]. MMPs play important roles in various physiological and pathological processes including developmental morphogenesis, tissue repair, skin aging, and tumor invasion. Expressions of some MMPs are regulated by diverse extracellular stimuli including UV, proinflammatory cytokines, and growth factors [11, 29, 31-33]. Certain MMPs, such as MMP-1, MMP-3, and MMP-9, are important mediators of tissue damage by UV irradiation in the human

skin *in vivo* [11, 12]. However, rodents lack the MMP-1 gene; therefore, MMP-1 is functionally replaced by MMP-13 [34-37]. These UV-induced MMPs causes loss of elastic fiber and collagen and finally leads to wrinkle formation [34].

Transient receptor potential cation channel subfamily V member 1 (TRPV1)

TRPV1 is a member of the nonselective cationic channel family [38, 39]. TRPV1 activation induces influx of calcium, which can be inhibited by a specific TRPV1 antagonist, capsazepine, and a nonselective TRPV1 antagonist, ruthenium red [40]. A previous report has shown that calcium influx via TRPV1 plays an important role in UV-induced MMP-1 expression in HaCaT cells. TRPV1 has been suggested to be the target for prevention of skin photoaging, which is commonly caused by UV exposure [38, 39]. UV irradiation has been shown to activate Src kinase and induce TRPV1 trafficking from the intracellular vesicles to the cell membrane within 15 minutes after UV irradiation. Subsequently, other kinases phosphorylate TRPV1 and lead to gate opening of the channel. Upon gating, calcium flows into the cell, and signal transduction follows. This leads to the induction of MMP-1 expression, which contributes to photoaging [41].

PURPOSES

Chapter I. Gasdermin C is Induced by Ultraviolet Light and Contributes to MMP-1 Expression via Activation of ERK and JNK Pathways.

The main objectives of this chapter are: to investigate the role of GSDMC in UV-induced MMP-1, MMP-3, and MMP-9 expressions in human skin keratinocytes and find out the molecular mechanisms involved in this process.

Chapter II: Ultraviolet Light-Induced Gasdermin C Expression is Mediated via TRPV1/Calcium/Calcineurin/NFATc1 Pathway.

The main objectives of this chapter are: to investigate the role of TRPV1 in UV-induced GSDMC expression and find out the signaling pathway involved in this process.

Chapter III: Cathepsin G Causes Ultraviolet Irradiation-induced Basement Membrane Damages in Hairless Mouse Skin.

The main objective of this chapter is to investigate the role of CTSG on basement membrane damages in chronically UV-irradiated hairless mouse skin.

Chapter IV: Screening of Cathepsin G Inhibitor using Plant Extracts and Drugs.

The main objective of this chapter is to find out the other substances (e.g. plant extracts, drugs) that are safe for clinical application in human skin instead of KPA.

CHAPTER I

Gasdermin C is Induced by Ultraviolet Light and Contributes to MMP-1 Expression via Activation of ERK and JNK Pathways

Materials and Methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Welgene (Gyeongsan, South Korea). Fetal Bovine Serum (FBS) was obtained from Hyclone (Logan, UT). Keratinocyte basal medium (MCDB153) and keratinocyte growth medium were purchased from Sigma-Aldrich (St. Louis, MO) and Clonetics (San Diego, CA), respectively. Antibiotics were obtained from Life Technologies (Rockville, MD). The MEK inhibitor PD98059 and the JNK inhibitor SP600125 were purchased from Calbiochem (San Diego, CA).

Cell culture and UV irradiation

An immortalized human keratinocyte cell line, HaCaT, and primary human skin keratinocytes established by outgrowth from foreskin biopsies of healthy donors were used. HaCaT cells were cultured in DMEM supplemented with penicillin (400 U/mL), streptomycin (50 mg/mL), and 10% FBS in a humidified 5% CO_2 atmosphere at 37°C. Primary human skin keratinocytes were cultured in keratinocyte growth medium supplemented with penicillin (400 U/mL) and streptomycin (50 mg/mL) in a humidified 5% CO_2 atmosphere at 37°C. Cultured primary human skin keratinocytes at passages 3-4 were used for the experiments. For UV irradiation, Philips TL 20W/12RS fluorescent sun lamps (Philips, Eindhoven, Netherlands) with emission spectrum between 275 and 380 nm (peak, 310–315 nm) were used as the UV source. The power output distribution of the UV

emission spectrum was 25.3% UVA1 (320–340 nm), 11.2% UVA2 (340–380 nm), 53.3% UVB (290– 320 nm), and 10.2% UVC (275–290 nm). A Kodacel filter (TA401/407; Kodak, Rochester, NY) was used to block UVC (< 290 nm). Before treatment, HaCaT cells were serum-starved for 48 hours in DMEM containing 0.25% FBS, while primary human skin keratinocytes were serum-starved for 24 hours in keratinocyte basal medium (MCDB153). HaCaT cells and primary human skin keratinocytes were washed with PBS twice and irradiated with UV (60 and 100 mJ/cm^2 respectively) in PBS. After UV irradiation, PBS was removed and replaced with DMEM without FBS for HaCaT cells and keratinocyte basal medium for primary human skin keratinocytes. Then cells were further incubated for the indicated times. For checking cell viability, cells were irradiated with UV (20, 40, 60, 80 and 100 mJ/cm^2 for HaCaT cells, and 60, 80, 100, 120, and 140 mJ/cm^2 for primary human skin keratinocytes). Then cell viability was determined at 48 hours after UV irradiation using EZ-CYTOX cell viability assay kit (Daeil Bio, Suwon, South Korea) according to the manufacturer's instructions. Briefly, HaCaT cells and primary human skin keratinocytes were treated with EZ-CYTOX reagent at 48 hours after UV irradiation, and cultured media were collected at 30 minutes after treatment. Then, the absorbance at 450/650 nm of the collected media was determined by microplate reader (Molecular Devices, Sunnyvale, CA).

Knockdown of GSDMC (Transfection with small interfering RNA (siRNA))

For knockdown of GSDMC, HaCaT cells or primary human skin keratinocytes were seeded and transfected with the negative control siRNA (scrambled siRNA) or the GSDMC-specific siRNA (Bioneer, Daejeon, Korea) using Lipofectamine® 2000 Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. After transfection, cells were serum-starved for 48 hours (HaCaT cells) or 24 hours (primary human skin keratinocytes), treated with UV, and then harvested at 24 and 48 hours for analysis of mRNA or protein.

Overexpression of GSDMC (Transfection with mammalian expression vector)

For overexpression of GSDMC, HaCaT cells or primary human skin keratinocytes were seeded and transfected with the control mammalian expression vector or the human GSDMC-containing mammalian expression vector using Lipofectamine® 2000 Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. The plasmid for human GSDMC (RC223817) was purchased from ORIGENE (Rockville, MD). After transfection, cells were serum-starved for 48 hours and then harvested for analysis of mRNA or protein.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from skin samples and cells using RNAiso Plus (Takara Bio, Shiga, Japan) and the same amount of RNA per each sample was used for the first-strand cDNA synthesis using First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. To estimate mRNA expression levels, quantitative real-time PCR was performed on a 7500 Real-time PCR System (Applied Biosystems, Foster City, CA) using SYBR *Premix Ex Taq*™ (Perfect (Perfect Real Time) (Takara Bio, Shiga, Japan) according to the manufacturer's instructions, with the following primer pairs: 36B4 (forward,5'-TGGGCTCCAAGCAGATGC-3'; reverse, 5'-GGCTTCGCTGGCTCCCAC-3'), GSDMC (forward,5'-TGCTCCCTCGAGTTTCAAAT-3'; reverse,5'-GGCTCTGGATCCAACAGTTT-3'), MMP-1 (forward,5'-ATTCTACTGATATCGGGGCTTTGA-3'; reverse,5'-ATGTCCTTGGGGTATCCGTGTAG-3'), MMP-3 (sense, 5'-CTC ACA GAC CTG ACT CGG TT-3'; antisense, 5'-CAC GCC TGA AGG AAG AGA TG-3'; MMP-9 (forward, 5'-TTG ACA GCG ACA AGA AGT GG-3'; reverse, 5'-GCC ATT CAC GTC GTC CTT AT-3'). The PCR conditions were 50°C for 2 minutes, 95°C for 2 minutes, followed by 40 cycles at 95°C for 15 second and 60°C for 1 minute. After being normalized versus 36B4, relative mRNA expression levels were determined.

Western blotting

Proteins from skin samples and cells were extracted using 1X RIPA lysis buffer (Merck Millipore, Billerica, MA) containing protease inhibitor mixture (Roche Applied Science, Rockford, IL) and phosphatase inhibitor mixture (Sigma-Aldrich, St. Louis, MO). Tissue extracts and cell lysates were centrifuged at 13,500 g, 4°C for 15 minutes, and supernatants were collected. Protein concentration of samples was determined by the Bicinchoninic acid assay reagent (Sigma-Aldrich, St. Louis, MO). To analyze secreted proteins including MMP-1, MMP-3 and MMP-9, culture media were collected. Proteins were loaded onto SDS–polyacrylamide gels, separated by gel electrophoresis and transferred to polyvinylidene difluoride membranes (Roche Applied Science Upper Bavaria, Germany). Membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% skim milk and incubated with rabbit polyclonal antibody against GSDMC (St John's Laboratory, London, United Kingdom), rabbit polyclonal antibody against MMP-1 (Lab Frontier, Seoul, Korea), mouse monoclonal antibody against MMP-3 (Neomarkers, Fremont, CA), rabbit polyclonal antibody against MMP-9 (Chemicon, Temecula, CA), p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38 MAPK, and p38 MAPK (Cell Signaling Technology, Beverly, MA), or a goat polyclonal antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies. Blots were visualized by enhanced chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA). Signal intensity was quantified by ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical analysis

Significance was determined using analysis of variance (ANOVA). Data are presented as mean ± SD. *P*-values of less than 0.05 were considered statistically significant.

Results

UV irradiation increases the expression of GSDMC in human skin keratinocytes

To study the function of GSDMC, especially the role of GSDMC in UV-induced expressions of various MMPs in human skin keratinocytes, we sought to examine whether GSDMC expression is regulated by UV irradiation in primary human skin keratinocytes and an immortalized human skin keratinocyte cell line, HaCaT cells. To do this, we first irradiated primary human skin keratinocytes and HaCaT cells with different doses of UV and then checked the cell viability (Figures 1a and 1b) and the expressions of GSDMC, MMP-1, MMP-3, and MMP-9 (Figures 1c-1j) at 48 hours after UV irradiation. We found that 60, 80, 100 mJ/cm^2 of UV for primary human skin keratinocytes and 20, 40, 60 mJ/cm^2 of UV for HaCaT cells did not decrease cell viability at 48 hours after UV irradiation. In contrast, 120, 140 mJ/cm^2 of UV for primary human skin keratinocytes and 80, 100 mJ/cm^2 of UV for HaCaT cells significantly decreased cell viability. We also found that 60 mJ/cm^2 of UV for primary human skin keratinocytes and 20, 40 mJ/cm^2 of UV for HaCaT cells did not increase the mRNA levels of GSDMC and MMP-1, whereas 80, 100, 120, 140 mJ/cm^2 of UV for primary human skin keratinocytes and 60, 80, 100 mJ/cm^2 of UV for HaCaT cells increased the mRNA levels of GSDMC and MMP-1 at 48 hours after UV irradiation. On the other hand, we observed that 60, 80 mJ/cm^2 of UV for primary human skin keratinocytes and 20, 40 mJ/cm^2 of UV for HaCaT cells did not increase the mRNA levels of MMP-3 and MMP-9, whereas 100, 120, 140 mJ/cm^2 of UV for primary human skin keratinocytes and 60, 80, 100 mJ/cm^2 of UV for HaCaT cells increased the mRNA levels of MMP-3 and MMP-9 at 48 hours after UV irradiation. Therefore, we chose 100 mJ/cm^2 of UV for primary human skin keratinocytes and 60 mJ/cm^2 of UV for HaCaT cells because those doses were the highest dose which increased the mRNA levels of GSDMC, MMP-1, MMP-3 and MMP-9 but did not decrease cell viability at 48 hours after UV irradiation. Then we further analyzed the mRNA and the protein levels of GSDMC,

at different time points. We found that both the mRNA and the protein levels of GSDMC were significantly increased at 24, 48, and 72 hours after UV irradiation in primary human skin keratinocytes (Figures 1k and 1l) and HaCaT cells (Figures 1m and 1n). Our results show that GSDMC is induced at both the mRNA and the protein levels by UV irradiation in both primary human skin keratinocytes and HaCaT cells.

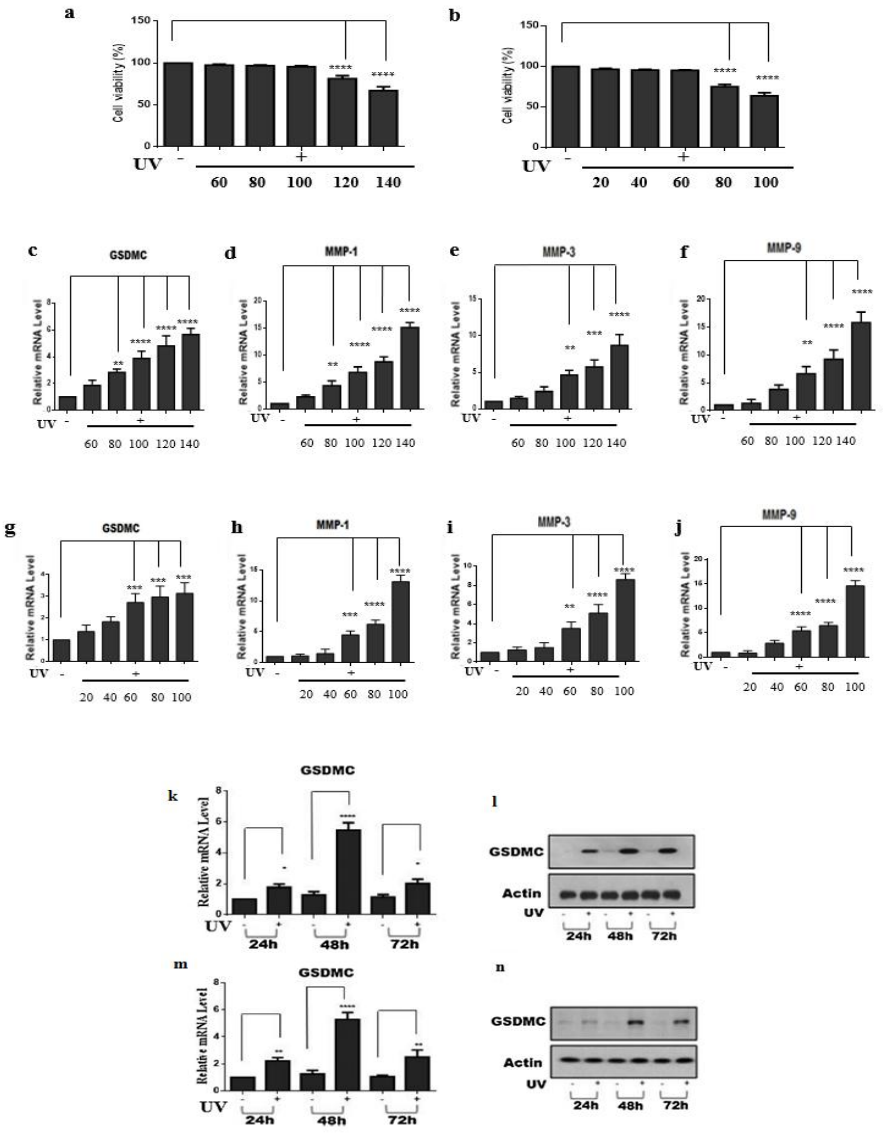

Figure 1. UV irradiation increases the expression of GSDMC in human skin keratinocytes

Primary human skin keratinocytes were irradiated with 60, 80, 100, 120 and 140 mJ/cm^2 doses of UV and HaCaT cells were irradiated with 20, 40, 60, 80, and 100 mJ/cm^2 doses of UV. Then cell viability for (a) primary human skin keratinocytes and (b) HaCaT cells were determined at 48 hours after UV irradiation. The mRNA levels of GSDMC, MMP-1, MMP-3, and MMP-9 for (c-f) primary human skin keratinocytes and (g-j) HaCaT cells were analyzed by qRT-PCR. Serum-starved primary human skin keratinocytes were irradiated with 100 mJ/cm2 of UV and harvested at 24, 48,

and 72 hours after UV irradiation for analysis of (k) mRNA and (l) protein. Serum-starved HaCaT cells were irradiated with 60 mJ/cm2 of UV and harvested at 24, 48, and 72 hours after UV irradiation for analysis of (m) mRNA and (n) protein. Data represents mean ± SD of relative mRNA expressions of GSDMC, MMP-1, MMP-3, and MMP-9 normalized to 36B4 (n=3). β-actin was used as a loading control (n=3). *P< 0.05, **P< 0.01, ***P< 0.001, and ****P< 0.0001 versus non UV-irradiated control.

GSDMC expression increases at relatively late time points after UV irradiation in HaCaT cells

Next, to understand the role of GSDMC in UV-induced MMP expressions in human skin keratinocytes, we examined the effect of UV on the expressions of GSDMC, MMP-1, MMP-3, and MMP-9 in more detail using time-course experiments. We irradiated serum-starved HaCaT cells with 60 mJ/cm^2 of UV, and then harvested cells at 2, 4, 8, 12, 16, 20 and 24 hours after UV irradiation. The mRNA and/or the protein levels of each gene were assessed by qRT-PCR and western blotting, respectively (Figure 2). The GSDMC expression started to increase at both the mRNA and the protein levels significantly from 20 hours after UV irradiation (Figures 2a and 2e). The mRNA expressions of MMP-1, MMP-3, and MMP-9 started to increase from 8 or 12 hours after UV irradiation and persisted until 24 hours after UV irradiation in HaCaT cells (Figures 2b, 2c, and 2d). We could detect the proteins of MMP-1, MMP-3, and MMP-9 in culture media from 8 or 12 hours after UV irradiation (Figure 2e). These results indicate that the expressions of GSDMC and MMPs start to increase at relatively late time points (from 8 hours or later) and persists until 24 hours and even 48 hours after UV irradiation in HaCaT cells (Figures 1, 2, and 3).

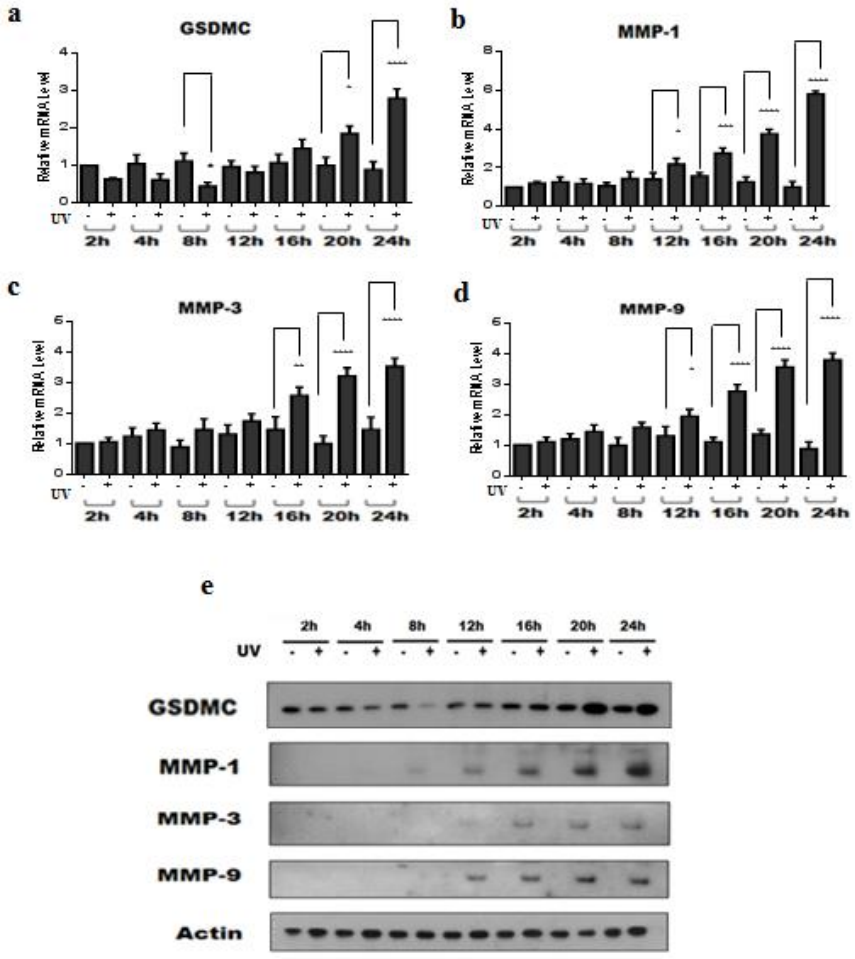

Figure 2. GSDMC expression increases at relatively late time points after UV irradiation in HaCaT cells

Serum-starved HaCaT cells were irradiated with 60 mJ/cm2 of UV and harvested at 2, 4, 8, 12, 16, 20 and 24 hours after UV irradiation. (a-d) The mRNA levels of GSDMC, MMP-1, MMP-3, and MMP-9 were analyzed by qRT-PCR. (e) The protein levels of GSDMC in cell lysates and MMP-1, MMP-3, and MMP-9 in culture media were analyzed by western blotting. Data represents mean ± SD of relative mRNA expressions of GSDMC, MMP-1, MMP-3, and MMP-9 normalized to 36B4 (n=3). β-actin was used as a loading control (n=3). *P< 0.05, **P< 0.01, ***P< 0.001, and ****P< 0.0001 versus non UV-irradiated control.

Knockdown of GSDMC reduces UV-induced expression of MMP-1, but not of MMP-3 and MMP-9 in HaCaT cells

Next, we investigated whether GSDMC can play any role in UV-induced expressions of MMP-1, MMP-3, and MMP-9. To do this, we first transfected HaCaT cells with negative control (scrambled) siRNA or GSDMC-specific siRNA. The transfected HaCaT cells were serum-starved for 48 hours, treated with UV, and then harvested at 24 and 48 hours post-UV treatment. We found that knockdown of GSDMC specifically and significantly decreased both the mRNA and the protein levels of MMP-1, but not of MMP-3 and MMP-9 (Figure 3). These results suggest that GSDMC plays a critical role in the regulation of UV-induced MMP-1 expression maybe at relatively late time points after UV irradiation in HaCaT cells.

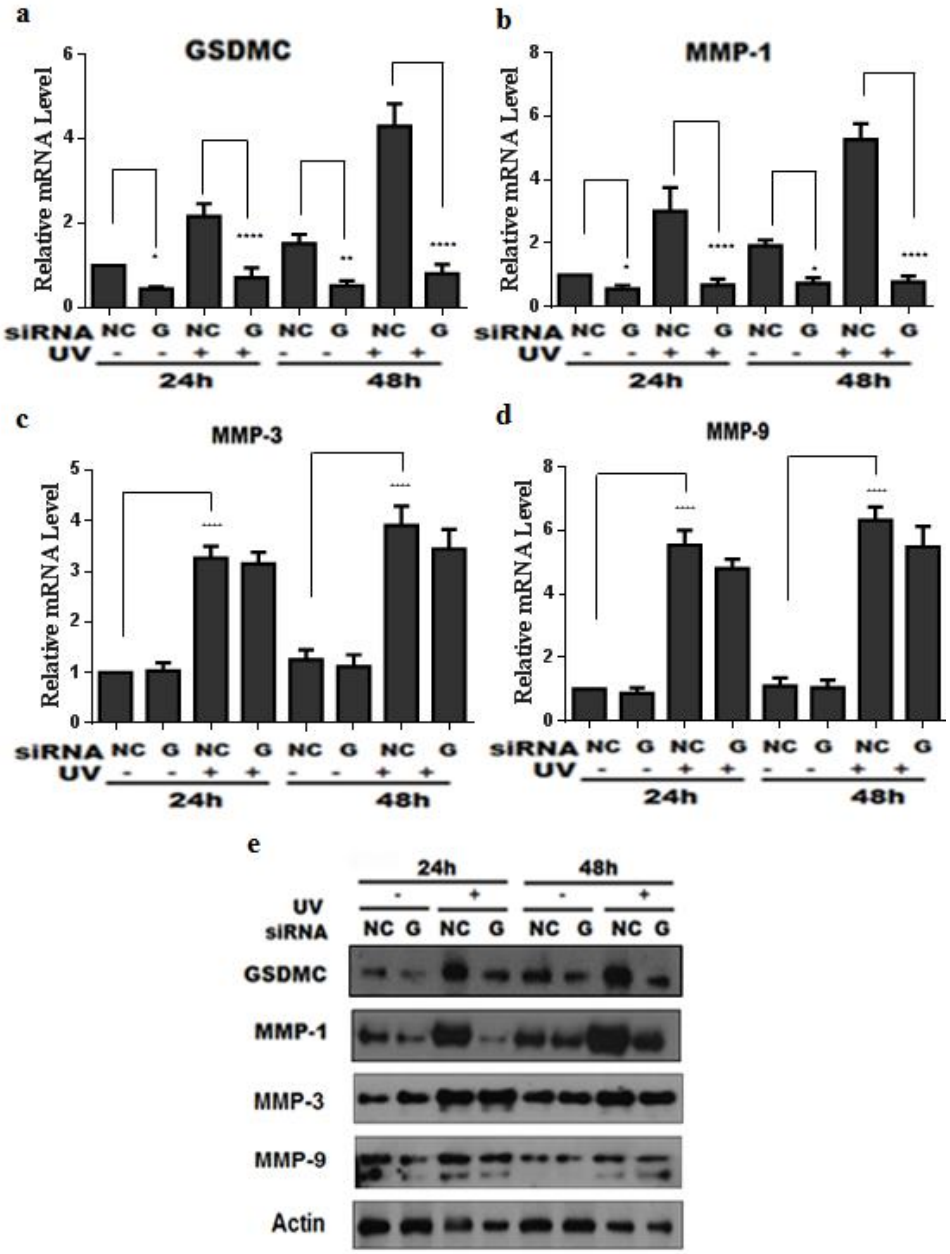

Figure 3. Knockdown of GSDMC reduces UV-induced expression of MMP-1, but not of MMP-3 and MMP-9 in HaCaT cells

HaCaT cells were transfected with the negative control siRNA or the GSDMC siRNA. The transfected cells were serum-starved for 48 hours, irradiated with 60 mJ/cm2 of UV, and then harvested at 24 and 48 hours after UV irradiation. (a-d) The mRNA levels of GSDMC, MMP-1,

MMP-3, and MMP-9 were analyzed by qRT-PCR. (e) The protein levels of GSDMC in cell lysates and of MMP-1, MMP-3, and MMP-9 in culture media were analyzed by western blotting. β-actin was used as a loading control (n=3). Data represents mean ± SD of relative mRNA expressions of GSDMC and MMP-1 normalized to 36B4 (n=3). β-actin was used as a loading control (n=3). *P< 0.05, **P< 0.01, ***P< 0.001, and ****P< 0.0001 versus non UV-irradiated control. NC: Negative control siRNA, G: GSDMC siRNA.

UV irradiation induces the activations of ERK and JNK not only at early time points but also at late time points

It is well known that the mitogen-activated protein kinases (MAPKs) play crucial roles on the MMP-1 expression induced by various stimuli including UV [10, 11, 42]. To elucidate how GSDMC regulates UV-induced MMP-1 expression, we examined the effect of UV on the activities of MAPKs in more detail using time-course experiments. We irradiated HaCaT cells with 60 mJ/cm^2 of UV and harvested cells at 2, 4, 8, 12, 16, 20, and 24 hours after UV irradiation. The activations of ERK, JNK, and p38 MAPK were assessed by detecting the phosphorylated forms of these MAPKs by western blotting. We found that there were two peaks in the activations of ERK and JNK, but not of p38 MAPK, during the time period (from 2 hours to 24 hours) used in this experiment. The first peak occurred at relatively early time points (2 and 4 hours after UV irradiation) and the second peak occurred at relatively late time points (24 hours after UV irradiation) (Figure 4).

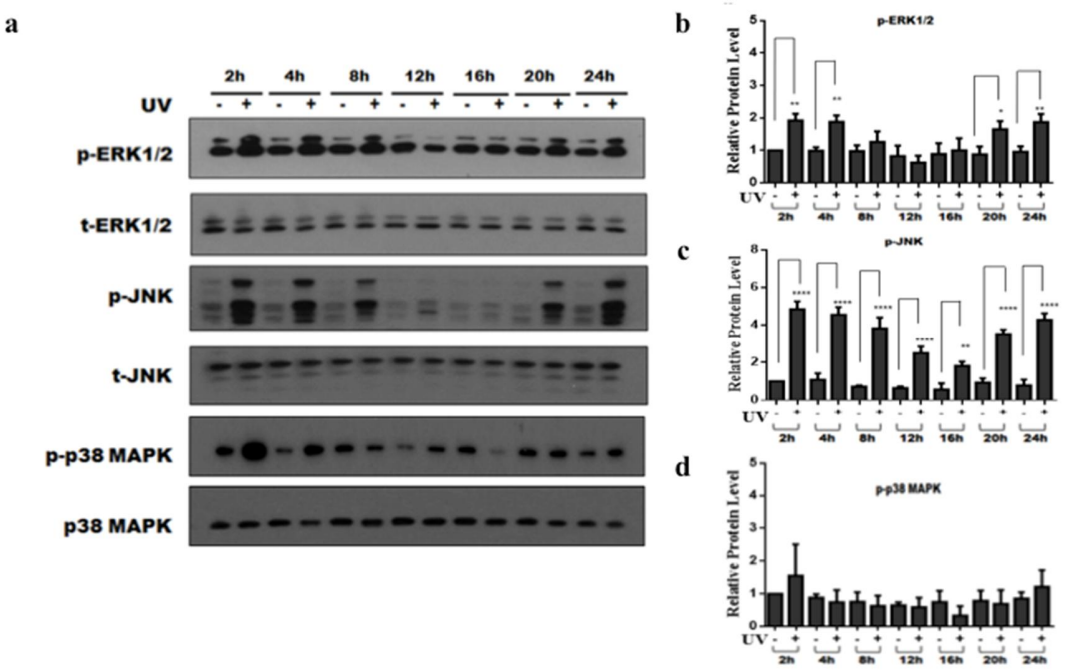

Figure 4. UV irradiation induces the activations of ERK and JNK not only at early time points but also at late time points

Serum-starved HaCaT cells were irradiated with 60 mJ/cm2 of UV and harvested at 2, 4, 8, 12, 16, 20 and 24 hours after UV irradiation. (a) Phosphorylated and total forms of ERK1/2, JNK, and p38 MAPK in cell lysates were analyzed by western blotting. (b-d) Relative protein levels were quantified by ImageJ software. Total forms of ERK, JNK, and p38 MAPK were used as loading controls (n=3). *P< 0.05, **P< 0.01, ***P< 0.001, and ****P< 0.0001 versus non UV-irradiated control.

Knockdown of GSDMC decreases UV-induced activations of ERK and JNK at late time points but not at early time points in HaCaT cells

Next, we investigated whether GSDMC can affect UV-induced activations of ERK, JNK or p38 MAPK. To do this, we first transfected HaCaT cells with negative control (scrambled) siRNA or GSDMC-specific siRNA. The transfected HaCaT cells were serum-starved for 48 hours, treated with UV, and then harvested at early time points (2 and 4 hours after UV irradiation) and late time points (24 and 48 hours after UV irradiation). We found that UV-induced activations of ERK and JNK but not of p38 MAPK were attenuated by knockdown of GSDMC expression at late time points, but not at early time points (Figure 5). Therefore, our results suggest that GSDMC plays an important role in UV-induced activations of ERK and JNK at relatively late time points after UV irradiation in HaCaT cells.

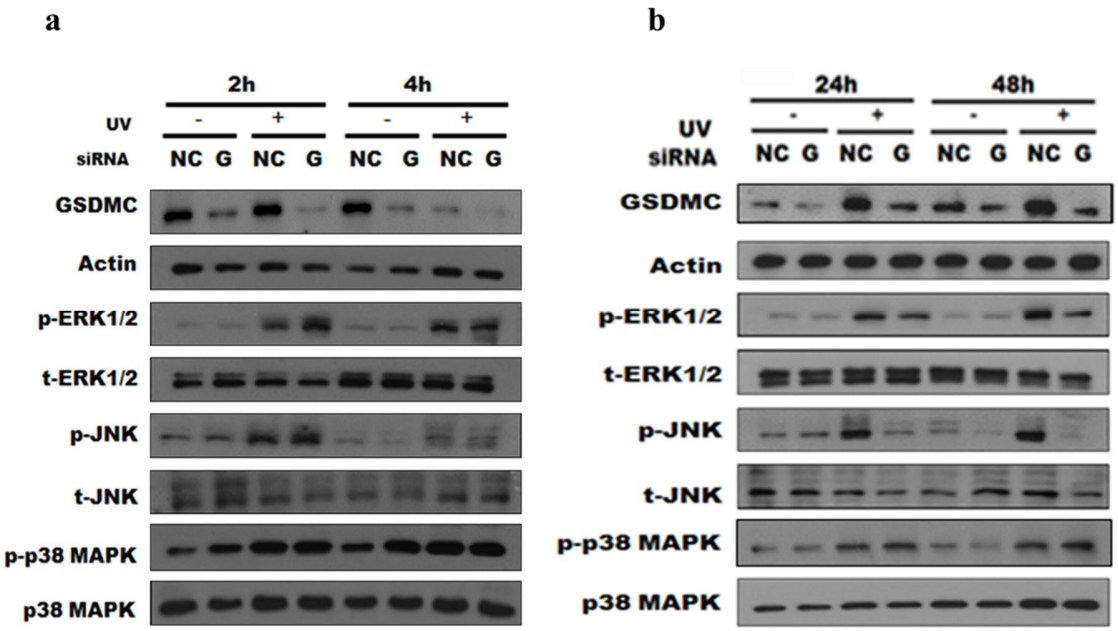

Figure 5. Knockdown of GSDMC decreases UV-induced activations of ERK and JNK at late time points but not at early time points in HaCaT cells

HaCaT cells were transfected with the negative control siRNA or the GSDMC siRNA. The transfected cells were serum-starved for 48 hours, irradiated with 60 mJ/cm2 of UV, and then harvested at 2, 4, 24 and 48 hours after UV irradiation. Phosphorylated and total forms of ERK1/2, JNK, p38 MAPK, and expressions of GSDMC in cell lysates were analyzed by western blotting. Total forms of ERK, JNK, p38 MAPK and β-actin were used as loading controls (n=3). NC: Negative control siRNA, G: GSDMC siRNA.

Overexpression of GSDMC increases MMP-1 expression through activation of ERK and JNK pathways in HaCaT cells

To complement the results obtained from our GSDMC knockdown experiments, we overexpressed GSDMC and examined expressions of MMPs and activations of MAPKs. HaCaT cells were transfected with the control vector or the mammalian expression vector containing GSDMC gene. Expressions of GSDMC protein and activations of MAPKs were analyzed in cell lysates, while expressions of MMP-1, MMP-3, and MMP-9 were analyzed in culture media. Overexpression of GSDMC increased the expression of MMP-1 but not of MMP-3 and MMP-9 and the activations of ERK and JNK but not of p38 MAPK. To confirm whether ERK and JNK pathways are genuinely involved in GSDMC-induced MMP-1 expression, we treated cells with DMSO as control (C), 50 μM PD98059 (PD) as a MEK1/2 inhibitor, and 20 μM SP600125 as a JNK inhibitor, after overexpression of GSDMC. We found that the MMP-1 protein levels increased by overexpression of GSDMC were significantly reduced by inhibition of ERK or JNK pathways (Figure 6).

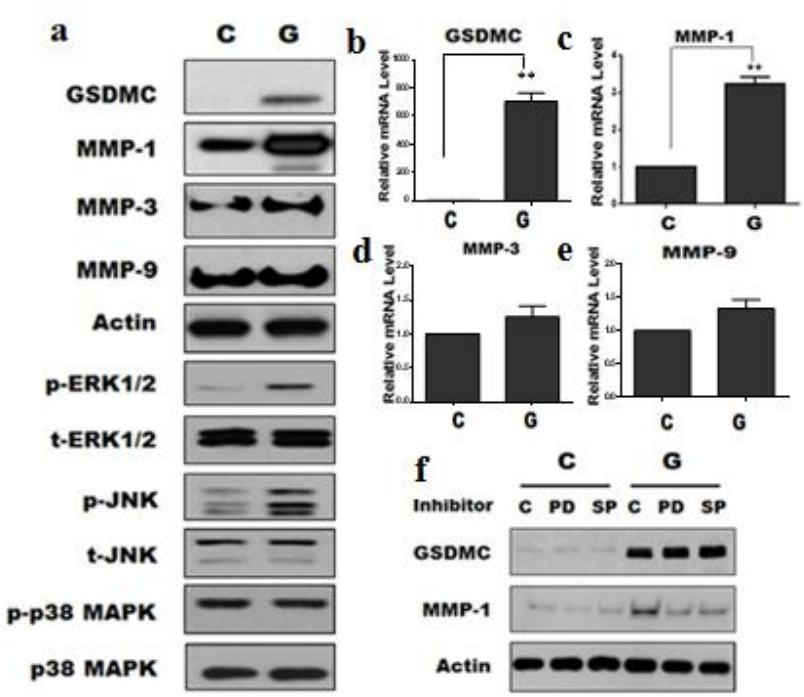

Figure 6. Overexpression of GSDMC increases MMP-1 expression through activation of ERK and JNK pathways in HaCaT cells

HaCaT cells were transfected with the control vector or the GSDMC expression vector. The transfected cells were serum-starved for 48 hours and then harvested for analysis of (a) protein and (b-e) mRNA, respectively. Protein levels of GSDMC in cell lysates and of MMP-1, MMP-3, and MMP-9 in culture media were analyzed by western blotting. Phosphorylated and total forms of ERK1/2, JNK, p38 MAPK in cell lysates were also analyzed by western blotting. Total form of ERK, JNK, p38 MAPK and β-actin were used as loading controls (n=3). GSDMC, MMP-1, MMP-3, and MMP-9 mRNA levels were analyzed by qRT-PCR (n=3). HaCaT cells were transfected with the control vector or the GSDMC expression vector, serum-starved for 24 hours, and then treated with the MEK or the JNK inhibitor. Cells were harvested at 48 hours after treatment. (f) Protein levels of GSDMC in cell lysates and of MMP-1 in culture media were analyzed by western blotting. Data represents mean ± SD of relative mRNA expressions of GSDMC, MMP-1, MMP-3, and MMP-9 normalized to 36B4 (n=3). Total forms of ERK, JNK, p38 MAPK or β-actin were used as

loading controls (n=3). **P <0.01 versus control vector. C: Control vector, G: GSDMC expression vector, DMSO as control (C), 50 μM PD98059 (PD) as a MEK inhibitor and 20 μM SP600125 (SP) as a JNK inhibitor.

GSDMC plays an important role in UV-induced MMP-1 expression through the activations of ERK and JNK also in primary human skin keratinocytes

Furthermore, we tried to determine whether knockdown and overexpression of GSDMC in primary human skin keratinocytes can also have similar results to those obtained in HaCaT cells. We found that knockdown of GSDMC decreased UV-induced expression of MMP-1 and activations of ERK and JNK at 24 and 48 hours after UV irradiation, while overexpression of GSDMC increased expression of MMP-1 and activations of ERK and JNK, in primary human skin keratinocytes (Figure 7). Taken together, our results indicate that GSDMC is increased at relatively late time points after UV irradiation and that this induction of GSDMC contributes to UV-induced MMP-1 expression in human skin keratinocytes.

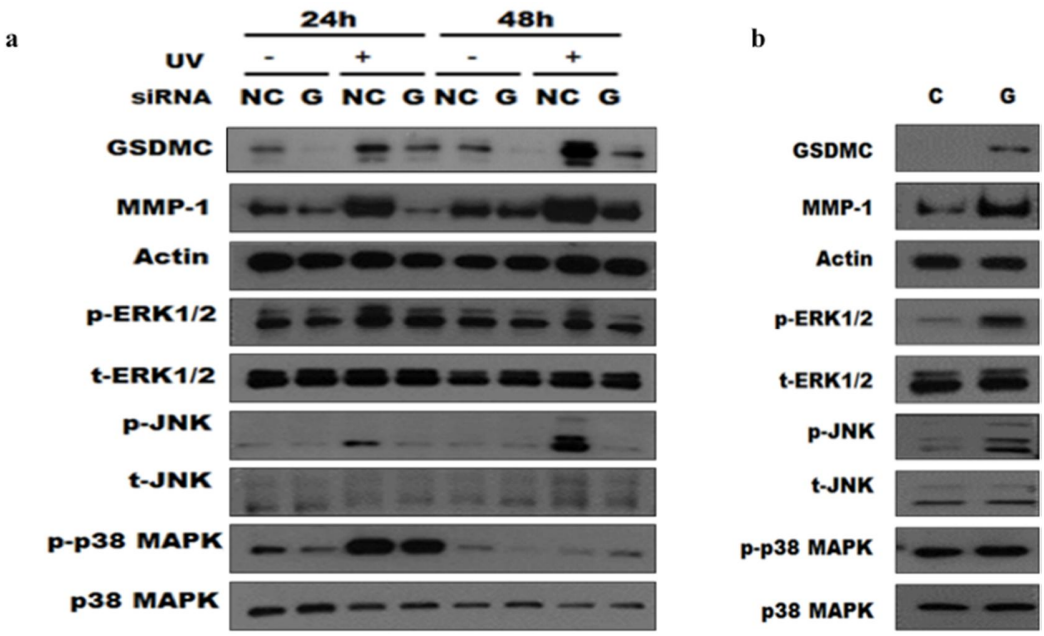

Figure 7. GSDMC plays an important role in UV-induced MMP-1 expression through the activations of ERK and JNK also in primary human skin keratinocytes

(a) Primary human skin keratinocytes were transfected with the negative control siRNA or the GSDMC siRNA. The transfected cells were serum-starved for 24 hours, irradiated with 100 mJ/cm^2 of UV, and then harvested at 24 and 48 hours after UV irradiation. (b) Primary human skin keratinocytes were transfected with the control vector or the GSDMC expression vector. The transfected cells were serum-starved for 48 hours and then harvested for analysis of protein. Phosphorylated and total forms of ERK1/2, JNK, p38 MAPK, and expressions of GSDMC in cell lysates and MMP-1 in culture media were analyzed by western blotting. Total form of ERK, JNK, p38 MAPK and β-actin were used as loading controls (n=3).

A schematic model of role of GSDMC in UV-induced MMP-1 expression

The ERK and JNK pathways are well known to play critical roles in UV-induced MMP-1 expression [10, 11]. However, how the activations of ERK and JNK pathways are regulated by UV is not completely understood. Our present results with the published works show that the activations of ERK and JNK pathways can occur not only at relatively early time points but also at relatively late time points after UV irradiation in human skin keratinocytes. The activations of ERK and JNK pathways at early time points after UV irradiation are due to activation of cell surface receptors such as EGFR [43, 44], whereas the activations of ERK and JNK pathways at late time points after UV irradiation are due to increase of GSDMC expression. Thus, the activations of ERK and JNK pathways at both early and late time points may contribute to full induction of MMP-1 expression by UV irradiation in human skin keratinocytes. Therefore, our results help us to better understand UV-induced activations of ERK and JNK pathways and MMP-1 expression in a time-dependent way and the role of GSDMC in the process (Figure 8).

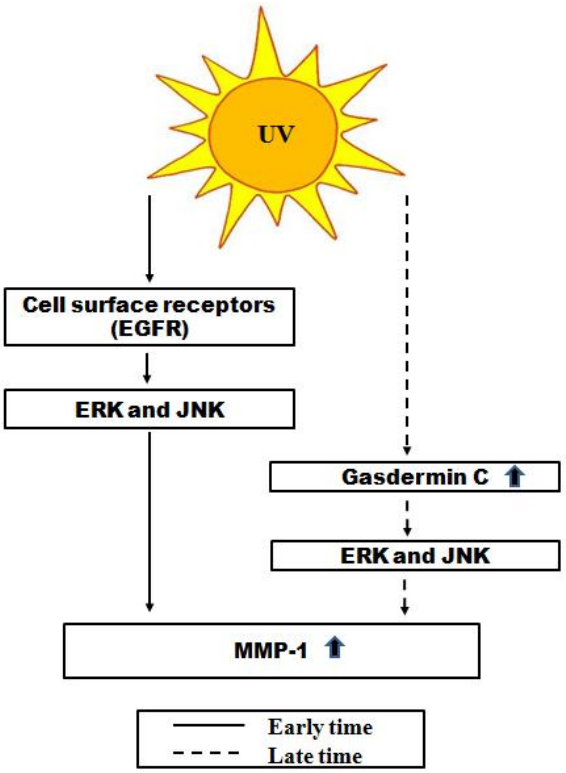

Figure 8. A schematic model of role of GSDMC in UV-induced MMP-1 expression

ERK and JNK pathways are well known to play critical roles in UV-induced MMP-1 expression. However, how the activations of ERK and JNK pathways are regulated by UV is not completely understood. Our present results with the published works indicate that the activations of ERK and JNK pathways can occur not only at relatively early time points but also at relatively late time points after UV irradiation in human skin keratinocytes. The activations of ERK and JNK pathways at early time points after UV irradiation are due to activation of cell surface receptors such as EGFR, whereas the activations of ERK and JNK pathways at late time points after UV irradiation are due to increase of GSDMC expression. The activations of ERK and JNK pathways at both early and late time points may contribute to full induction of MMP-1 expression by UV irradiation in human skin keratinocytes.

CHAPTER II

Ultraviolet light-induced Gasdermin C expression is mediated via TRPV1/calcium/ calcineurin/NFATc1 pathway

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), was purchased from Welgene, Inc. (Gyeongsan, Gyeongsangbuk, Korea). Calcium-free DMEM was obtained from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Fetal Bovine Serum (FBS) was purchased from HyClone (GE Healthcare Life Sciences, Logan, UT, USA). Keratinocyte basal medium MCDB 153 was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and keratinocyte growth medium was purchased from Clonetics Corp. (San Diego, CA, USA). Antibiotics (penicillin and streptomycin) and TRIzol reagent were obtained from Thermo Fisher Scientific, Inc. Capsazepine, ruthenium red and capsaicin were purchased from Sigma-Aldrich; Merck KGaA. Expression plasmids for the wild-type (pMX-NFATc1-WT) or a constitutively active form of NFATc1 (pMSCV-NFATc1-CA) were kindly provided by Dr Hong-Hee Kim (Department of Cell and Developmental Biology, Seoul National University, Seoul, Korea).

Cell culture and treatments

An immortalized human keratinocyte cell line, HaCaT, was purchased from CLS Cell Lines Service GmbH (Eppelheim, Germany). Primary human skin keratinocytes were cultured from foreskin of healthy donors. HaCaT cells were cultured in DMEM supplemented with glutamine (2 mM), penicillin (400 U/ml), streptomycin (50 mg/ml) and 10% FBS in a humidified 5% CO2 atmosphere

at 37˚C. Primary human skin keratinocytes were cultured in keratinocyte growth medium supplemented with penicillin (400 U/ml) and streptomycin (50 mg/ml) in a humidified 5% CO2 atmosphere at 37˚C; cultured primary human skin keratinocytes at passages 3-4 were used. For treatments, HaCaT cells were cultured to 80% confluence and serum-starved for 24 hours in DMEM without FBS, and primary human skin keratinocytes were serum-starved for 24 hours in MCDB 153. HaCaT cells and primary human skin keratinocytes were washed with phosphate-buffered saline (PBS) twice; subsequently, HaCaT cells were irradiated with UV at 60 mJ/cm2 and primary human skin keratinocytes were irradiated with UV at 100 mJ/cm2 in PBS. UV irradiation was performed with Philips TL 20W/12RS fluorescent sun lamps (Philips Medical Systems B.V., Eindhoven, The Netherlands) with an emission spectrum between 275 and 380 nm (peak, 310-315 nm); a Kodacel filter TA401/407 (Kodak, Rochester, NY, USA) was used to block UVC of wavelength below 290 nm. UV irradiation intensity was measured with a Model 585100 UV meter from Herbert Waldmann GmbH & Co. KG (Villingen-Schwenningen, Germany). Following UV irradiation, PBS was removed and replaced with DMEM without FBS for HaCaT cells and keratinocyte basal medium for primary human skin keratinocytes, and cells were further incubated for 24 hours. When required, specific TRPV1 antagonist (capsazepine or ruthenium red) or specific TRPV1 agonist (capsaicin) was added 30 minutes prior to UV irradiation, and treated again with specific TRPV1 antagonist (capsazepine or ruthenium red) for 24 hours following UV irradiation; calcineurin inhibitor (cyclosporine A) was added immediately following UV irradiation and cells were incubated for 24 hours. To investigate the role of extracellular calcium in UV irradiation, HaCaT cells were serum-starved for 24 hours and cultured in either calcium-free DMEM or calcium-containing DMEM for 30 minutes prior to UV irradiation. Fresh corresponding culture medium was added, and the cells were further incubated for 24 hours. Each experiment was repeated three times. The medical ethical committee at Seoul National University approved the study protocol, and written informed consent was received from the guardians of participants. The study was conducted according to the Declaration of Helsinki principles.

Knockdown of NFATc1 (Transfection with small interfering RNA (siRNA))

For knockdown of NFATc1, cultured HaCaT cells were seeded and maintain until approximately 80% confluency, and subsequently transfected with the scrambled negative control siRNA (siNC) or a NFATc1-specific siRNA (siNFATc1; 5'-CCAAGG UCAUUUUCGUGGA-3'; Bioneer Corporation, Daejeon, Korea) at 100 pmol using Lipofectamine® 2000 Reagent (Invitrogen: Thermo Fisher Scientific, Inc.) in a humidified 5% CO2 atmosphere at 37˚C for 6 hours, according to manufacturer's instruction. The concentration of siRNA primer pairs was determined by dose response (data not shown). Following transfection, cells were serum-starved for 24 hours, treated with UV and incubated for an additional 24 hours. Cells were harvested for analysis of mRNA or protein. Each experiment was repeated three times.

Overexpression of NFATc1 (Transfection with mammalian expression vector)

For overexpression of NFATc1, cultured HaCaT cells were seeded and maintain until approximately 80% confluency, and subsequently transfected with the control empty vector or the mammalian expression vectors containing either pMX-NFATc1-WT or pMSCV-NFATc1-CA using Lipofectamine 2000 Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) at 2 μg in a humidified 5% CO2 atmosphere at 37˚C for 6 hours, according to the manufacturer's protocol. The concentration of vectors was determined by dose response (data not shown). Following transfection, cells were serum-starved for 24 hours, treated with UV and incubated for an additional 24 hours. Cells were harvested for analysis of mRNA or protein. Each experiment was repeated three times.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from HaCaT cells at ~70% confluency using the TRIzol method, according to the manufacturer's protocol. The quality of isolated RNA samples were measured by electrophoresis in 1% agarose gels (data not shown). Total RNA (1 μg) was used in a 20 μl reaction for first-strand cDNA synthesis using First Strand cDNA Synthesis Kit (MBI Fermentas; Thermo

Fisher Scientific, Inc.), according to the manufacturer's protocol. cDNA was subjected to amplification reactions using a 7500 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Premix Ex Taq, Perfect Real-time (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol, with the following primer pairs: 36B4, forward 5'-TGGGCTCCAAGCAGATGC-3', reverse 5'-GGCTTCGCT GGCTCCCAC-3'; GSDMC, forward 5'-TGCTCCCTCGAG TTTCAAAT-3', reverse 5'-GGCTCTGGATCCAACAGT TT-3'. PCR thermocycling conditions were as follows: 50˚C for 2 minutes and 95˚C for 2 minutes, followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. Relative mRNA expression levels were normalized to 36B4 and relative expression levels of the target gene were calculated using the 2-ΔΔCq method [45]. Each experiment was repeated three times.

Western blotting

Western blot analysis was performed by extracting proteins from HaCaT cells and primary human skin keratinocytes at ~70% confluency using Radioimmunoprecipitation Assay Lysis Buffer (EMD Millipore, Billerica, MA, USA) mixed with protease inhibitor mixture (Roche Applied Science, Penzberg, Germany) and phosphatase inhibitor mixture (Sigma-Aldrich; Merck KGaA). Cell lysates were centrifuged at 13,500 x g at 4˚C for 15 minutes, and supernatants were collected. The total cell extract protein concentration was quantified by the Bicinchoninic Acid assay reagent (Sigma-Aldrich; Merck KGaA). Equal amounts of protein, 20 µg per well, were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Roche Applied Science). Following blocking for 1 hour in 5% skim milk diluted with Tris-buffered saline containing 0.1% Tween-20, the membranes were incubated overnight with primary antibodies (1:1,000) at 4˚C with rabbit polyclonal antibody against GSDMC (cat. no. STJ93220; St. John's Laboratory, London, United Kingdom), mouse monoclonal antibody against NFATc1 (7A6; cat. no. sc-7294; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and goat polyclonal antibody against β-actin (I-19; cat. no. sc-1616; Santa Cruz Biotechnology, Inc.); β-actin was used as a loading control. The membranes

were washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit (sc-2004), goat anti-mouse (sc-2005) or mouse anti-goat (sc-2354) immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA) as secondary antibodies (1:5,000) for 1 hour in room temperature. Immunoreactive bands were visualized using the Enhanced Chemiluminescence Detection System (Thermo Fisher Scientific, Inc.). Signal intensity was measured by ImageJ software version 1.51w (National Institutes of Health, Bethesda, MD). Protein expression levels were normalized to β-actin. Each experiment was repeated three times.

Statistical analysis

Significance was determined using ANOVA. Data are presented as mean ± SD. *P*-values of less than 0.05 were considered statistically significant.

Results

TRPV1 plays an important role in UV-induced GSDMC expression in human skin keratinocytes

A number of previous studies have reported that TRPV1 or GSDMC play important roles in UV-induced MMP-1 expression [39, 46, 47]. However, TRPV1 activation is induced at relatively early time points [39], whereas GSDMC expression is induced at relatively late time points, following UV irradiation [46]. Therefore, whether early activation of TRPV1 played a role in late induction of GSDMC expression following UV irradiation was examined. Serum-starved HaCaT cells were pre-treated with different TRPV1 inhibitors, either capsazepine (a specific TRPV1 antagonist) or ruthenium red (a non-selective TRPV1 antagonist), irradiated with UV and subsequently treated again with capsazepine or ruthenium red. Cells cultured with either TRPV1 inhibitor exhibited a reduction in UV-induced expression of GSDMC in a dose-dependent manner (Figures 9a and 9b). Furthermore, to confirm whether TRPV1 was involved in UV-induced GSDMC expression, HaCaT cells were treated with capsaicin (a specific TRPV1 agonist) [48-50] and GSDMC expression was examined. The results demonstrated that capsaicin treatment increased GSDMC expression in a dose-dependent manner (Figure 9c). In addition, whether the induction of GSDMC expression in primary human skin keratinocytes had similar effects to those obtained for HaCaT cells was examined; similarly, capsazepine and ruthenium red treatments inhibited UV-induced expression of GSDMC (Figures 9d and 9e), whereas capsaicin treatment increased GSDMC expression (Figure 9f) in primary human skin keratinocytes in a dose-dependent manner. Taken together, these results suggested that TRPV1 may play a crucial role in UV-induced GSDMC expression in human skin keratinocytes.

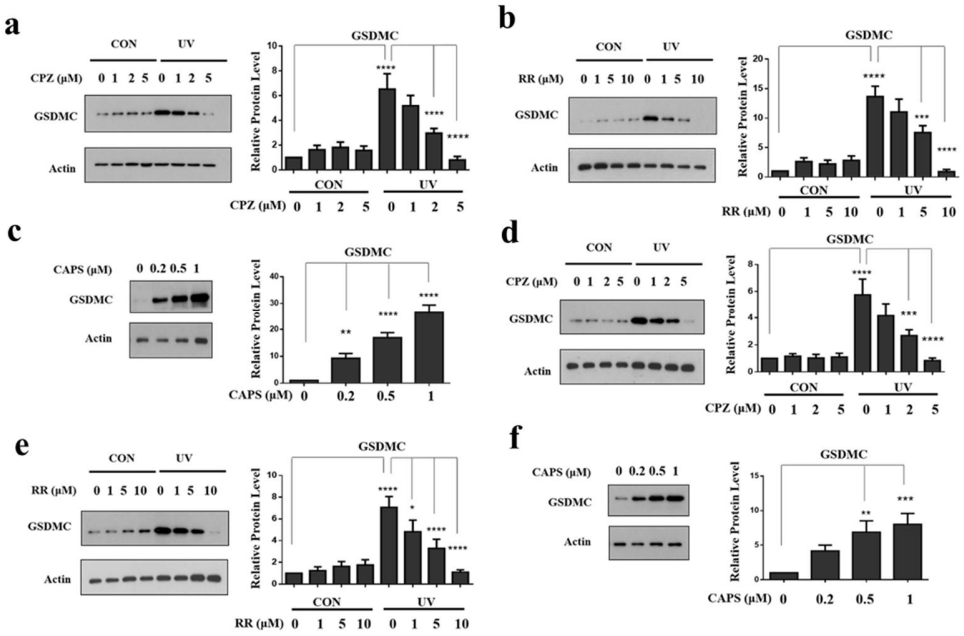

Figure 9. TRPV1 plays an important role in UV-induced GSDMC expression in human skin keratinocytes

(a and b) HaCaT cells were serum-starved for 24 hours. Following pre-treatment with (a) CPZ or (b) RR for 30 minutes, cells were irradiated with UV and fresh media containing the corresponding inhibitor were added and cells were incubated for an additional 24 hours. (c) HaCaT cells were serum-starved for 24 hours and treated with CAPS at the various concentrations for 24 hours. (d and e) Primary human skin keratinocytes were serum starved for 24 hours. Following pretreatment with (d) CPZ or (e) RR for 30 minutes, cells were irradiated with UV and fresh media containing the corresponding inhibitor were added and cells were incubated for an additional 24 hours. (f) Primary human skin keratinocytes were serum-starved for 24 hours and treated with CAPS at the various concentrations for 24 hours. GSDMC protein expression was analyzed by western blotting and relative protein levels were quantified by ImageJ software; β-actin was used as a loading control. Data are presented as the mean ± SD (n=3). *P< 0.05, **P< 0.01, ***P< 0.001, and ****P< 0.0001.

CAPS, Capsaicin; CPZ, Capsazepine; CON, control non-UV irradiated; GSDMC, gasdermin C; RR: Ruthenium red; UV, ultraviolet irradiated.

UV-induced GSDMC expression is calcium-dependent in HaCaT cells

TRPV1 acts as a non-selective cation channel and the activation of TRPV1 leads to calcium influx [39, 47, 51]. As TRPV1 may be involved in UV-induced GSDMC expression, the role of extracellular calcium on UV-induced GSDMC expression was examined. Serum-starved HaCaT cells were pre-incubated in either calcium-containing DMEM or calcium-free DMEM, irradiated with UV and further incubated for 24 hours in the corresponding media. UV-induced GSDMC mRNA and protein expression levels were notably reduced in calcium-free DMEM, compared with expression in calcium-containing DMEM (Figures 10a and 10b). However, calcium supplementation into calcium-free DMEM led to increased GSDMC mRNA and protein expression levels following UV irradiation in a dose-dependent manner (Figures 10c and 10d). These results indicated that UV-induced GSDMC expression may be calcium-dependent.

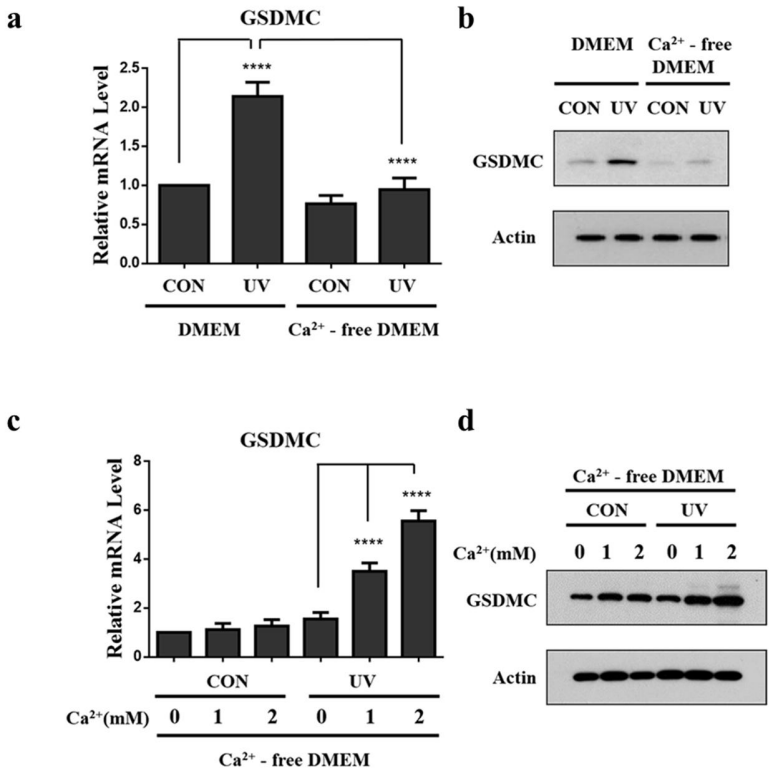

Figure 10. UV-induced GSDMC expression is calcium-dependent in HaCaT cells

(a and b) HaCaT cells were serum-starved, pre-incubated in either calcium-containing DMEM or calcium-free DMEM, irradiated with UV and incubated for an addition 24 hours in fresh calcium-containing DMEM or calcium-free DMEM. (c and d) Alternatively, calcium was added at various concentrations in calcium-free DMEM prior to UV irradiation. Fresh corresponding culture medium containing calcium was added and the cells were further incubated for 24 hours. GSDMC mRNA expression levels were analyzed by qRT-PCR. Data represents mean ± SD of relative mRNA expressions of GSDMC normalized to 36B4 (n=3). ****P< 0.0001. GSDMC protein expression levels were analyzed by western blotting; β-actin was used as a loading control (n=3). CON, control non-UV irradiated; DMEM, Dulbecco's modified Eagle's medium; GSDMC, gasdermin C; UV, ultraviolet irradiated.

Calcineurin pathway plays a crucial role in UV-induced GSDMC expression in HaCaT cells

Calcineurin is a calcium and calmodulin-dependent serine/threonine protein phosphatase [52, 53]. As the present results indicated that UV-induced GSDMC expression may be calcium-dependent, whether calcineurin was involved in UV-induced GSDMC expression was examined. HaCaT cells were irradiated with UV and subsequently treated with cyclosporine A (a calcineurin inhibitor) at the various concentrations (0, 5, 10 or 20 μM) for 24 hours. UV-induced GSDMC mRNA and protein expression levels were notably inhibited by cyclosporine A in a dose-dependent manner, in control non-UV-irradiated cells and in UV-irradiated cells (Figures 11a and 11b). These results indicated that the calcineurin pathway may play an important role in UV-induced GSDMC expression.

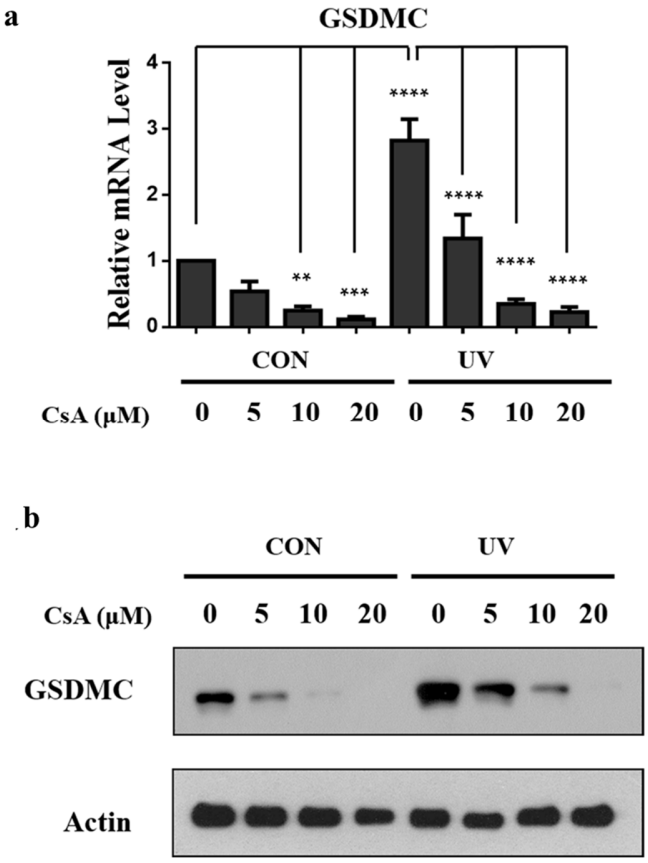

Figure 11. Calcineurin pathway plays a crucial role in UV-induced GSDMC expression in HaCaT cells

(a and b) Serum-starved HaCaT cells were irradiated with UV, incubated with CsA for 24 hours. (a) GSDMC mRNA expression levels were analyzed by qRT-PCR. Data represents mean ± SD of relative mRNA expressions of GSDMC normalized to 36B4 (n=3). **P< 0.01, ***P< 0.001, and ****P< 0.0001. (b) GSDMC protein expression levels were analyzed by western blotting; β-actin was used as a loading control (n=3). CON, control non-UV irradiated; DMEM, CsA, Cyclosporin A; GSDMC, gasdermin C; UV, ultraviolet irradiated.

UV-induced GSDMC expression is mediated through NFATc1 in HaCaT cells

Whether NFATc1 was involved in UV-induced GSDMC expression was examined, as calcineurin is known to activate the NFATc family members by dephosphorylating them, and NFATc1 was reported to be expressed in HaCaT cells [54-57]. HaCaT cells were transfected with either siNC or siNFATc1, serum-starved for 24 hours, treated with UV and further incubated for 24 hours. NFATc1 protein expression levels were increased by UV irradiation in the siNC- and in the siNFATc1-treated cells (Figure 12a), which was consistent with previous reports indicating that UV induces NFATc1 expression [58]. However, the knockdown of NFATc1 expression notably reduced the basal and the UV-induced levels of GSDMC protein and mRNA expression (Figures 12a and 12b). In addition, HaCaT cells were transfected with a control empty vector or with either a mammalian expression vectors containing a wild-type or a constitutively active form of NFATc1 gene. The vector-transfected HaCaT cells were serum-starved for 24 hours, treated with UV and further incubated for 24 hours. Overexpression of the wild-type of NFATc1 notably increased UV-induced GSDMC expression and the overexpression of a constitutively active form of NFATc1 also notably increased both basal levels and UV-induced levels of GSDMC mRNA and protein expression (Figures 12c and 12d). Taken together, these results indicated that UV-induced GSDMC expression may be mediated through NFATc1 in HaCaT cells.

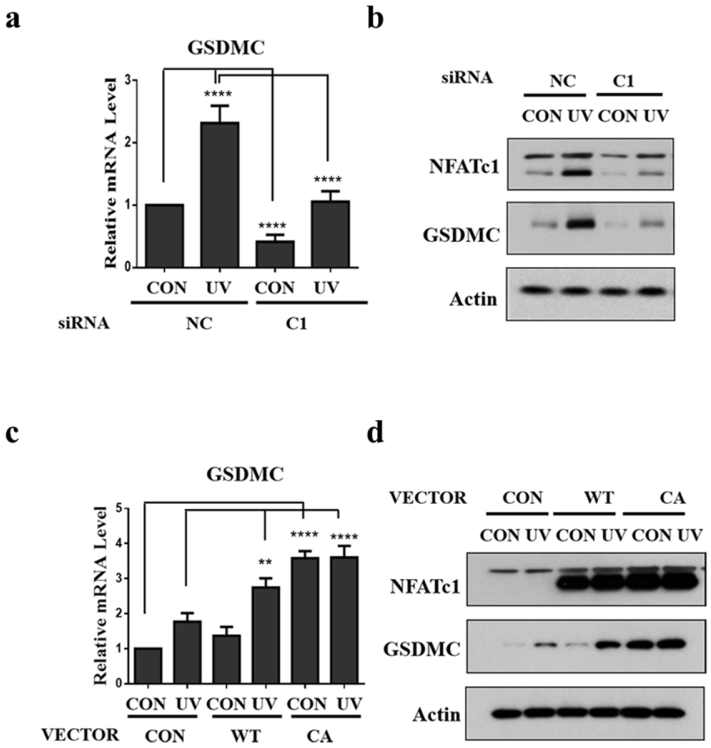

Figure 12. UV-induced GSDMC expression is mediated through NFATc1 in HaCaT cells

(a and b) HaCaT cells were transfected with siNC or siNFATc1, serum-starved, irradiated UV and harvested at 24 hours following UV irradiation. (a) NFATc1 and GSDMC protein expression levels were analyzed by western blotting; β-actin was used as a loading control; n=3. (b) GSDMC mRNA expression levels were analyzed by qRT-PCR. (c and d) HaCaT cells were transfected with the CTRL empty vector, NFATc1-WT or NFATc1-CA overexpression vectors. The cells were serum-starved, irradiated with UV and harvested at 24 hours following UV irradiation. (c) GSDMC mRNA expression levels were analyzed by qRT-PCR. (d) NFATc1 and GSDMC protein expression level were analyzed by western blotting; β-actin was used as a loading control (n=3). Data represents mean ± SD of relative mRNA expressions of GSDMC normalized to 36B4 (n=3). **P< 0.01, ****P< 0.0001. CA, Constitutively active NFATc1 expression vector; CON, control non-UV irradiated; CTRL, empty vector control; GSDMC, gasdermin C; NFATc1, nuclear factor of

activated T-cells, cytoplasmic 1; si, small interfering RNA; siNC, negative control siRNA; UV, ultraviolet irradiated; WT, wild-type NFATc1 expression vector.

A schematic model of the signaling pathways involved in UV-induced GSDMC and MMP-1 expressions

Expression of MMP-1 by UV can be regulated through various factors and signaling pathways. The activation of cell surface receptors including EGFR by UV induces signal transduction cascades and activates various signaling pathways such as ERK and JNK pathways that are known to be important for MMP-1 expression. In addition to these cell surface receptors, TRPV1 can also be activated by UV. The activation of TRPV1 turns on the calcium/calcineurin/NFATc1 signaling pathway, which leads to increase GSDMC expression. The increase of GSDMC expression activates ERK and JNK pathways, which leads to the induction of MMP-1 expression (Figure 13).

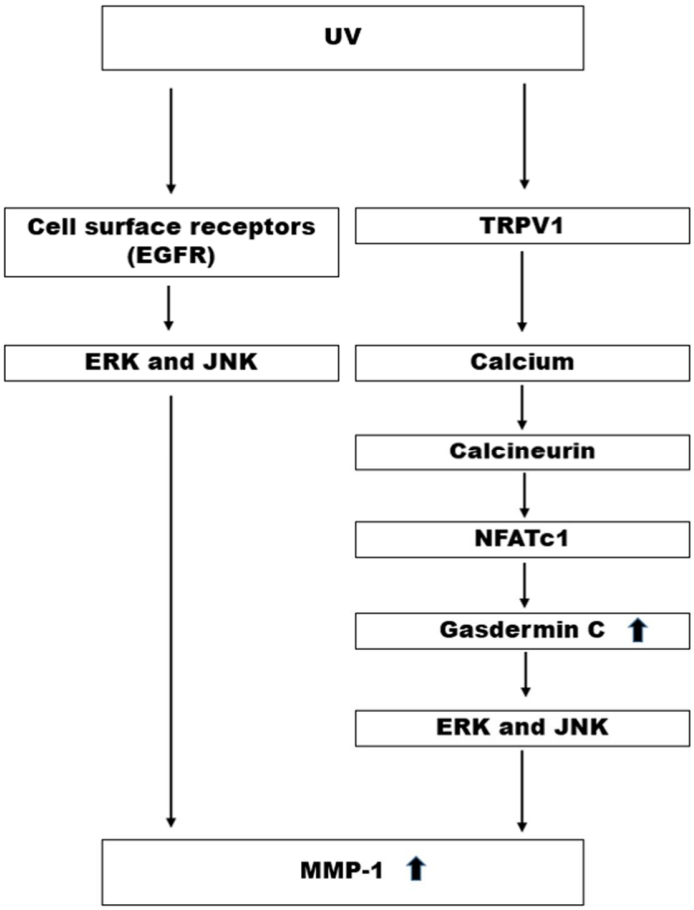

Figure 13. A schematic model of the signaling pathways involved in UV-induced GSDMC and MMP-1 expressions

UV-induced expression of MMP-1 may be regulated through various factors and signaling pathways. The activation of cell surface receptors, including EGFR, by UV induces signal transduction cascades and activates various signaling pathways, such as ERK and JNK pathways, that are known to be important for MMP-1 expression. In addition to these cell surface receptors, TRPV1 is also be activated by UV, which turns on the calcium/calcineurin/NFATc1 signaling pathway and leads to increased GSDMC expression. The increase of GSDMC expression activates ERK and JNK pathways, ultimately inducing MMP-1 expression. EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; GSDMC, gasdermin C; JNK, c-Jun N-terminal

kinase; MMP-1, matrix metalloproteinase 1; NFATc1, nuclear factor of activated T-cells, cytoplasmic 1; TRPV1, transient receptor potential cation channel subfamily V member 1; UV, ultraviolet irradiation.

CHAPTER III

Cathepsin G Causes Ultraviolet Irradiation-induced Basement Membrane Damages in Hairless Mouse Skin

Materials and Methods

Materials

Cathepsin G (CTSG) inhibitor, β-keto-phosphonic acid (KPA), was purchased from Calbiochem (Darmstadt, Germany) and diluted in dimethyl sulfoxide (DMSO) following manufacturer's instructions.

Animals and UV irradiation

Hairless female mice, 5-6 weeks old, were purchased from Orient Bio Inc. (Seoul, Korea). All mice were housed according to the group, seven mice per cage, under controlled conditions. All experiments were approved by the Institutional Animal Care and Use Committees (IACUC No. 14-0225-C1A0) at Seoul National University.

UV irradiation was performed using Philips TL20W/12RS UV lamps (Philips, Eindhoven, Netherlands) with emission spectrum between 275 and 380 nm (peak, 310–315 nm). A Kodacel filter (TA401/407; Kodak, Rochester, NY) was used to remove UVC wavelengths below 290 nm. The dorsal skin of hairless mice was irradiated 3 times/week (Monday, Wednesday, and Friday). Chronic UV irradiation was performed with 300 mJ/cm^2 for the first 2 weeks, 450 mJ/cm^2 for the next 2 weeks, and 600 mJ/cm^2 for the last 4 weeks. Mice were divided into five groups: non-UV-irradiated control mice, non-UV-irradiated and KPA (0.025%)-treated mice, UV-irradiated mice,

UV-irradiated and KPA (0.005%)-treated mice, UV-irradiated and KPA (0.025%)-treated mice. KPA was applied as described previously [24]. KPA (0.005% or 0.025%) were topically applied to the dorsal skin immediately after each session of UV irradiation to avoid UV absorption by KPA.

Western blotting

Hairless mice were sacrificed and the dorsal skin was collected. Protein from mouse skin samples were extracted using RIPA lysis buffer (Merck Millipore, Billerica, MA) containing phosphatase inhibitor mixture (Sigma-Aldrich, St. Louis, MO) and protease inhibitor mixture (Roche Applied Science, Rockford, IL). Mouse skin tissue extracts were centrifuged at 13,500*g*, 4°C for 15 minutes, and supernatants were collected. Protein concentrations in samples were measured using the Bicinchoninic Acid assay (Sigma-Aldrich, St. Louis, MO). Proteins were loaded onto SDS–polyacrylamide gels, separated by gel electrophoresis and transferred to polyvinylidene difluoride membranes/PVDF (Roche Applied Science Upper Bavaria, Germany). Membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% skim milk and incubated with rabbit polyclonal antibody against CTSG (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal antibody against MMP-13 (Thermo Fisher Scientific, Waltham, MA), and goat polyclonal antibody against β-actin (Santa Cruz Biotechnology, Santa Cruz, CA). Horseradish peroxidase-conjugated anti-rabbit, anti-mouse, or anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies, respectively. Enhanced chemiluminescence detection system (Thermo Fisher Scientific, Waltham, MA) was used to detect bands. Signal intensity was quantified by ImageJ software (National Institutes of Health, Bethesda, MD).

Immunofluorescence (IF) staining

The dorsal skin of hairless mice were embedded in OCT compound (Tissue-Tek®, Torrance, CA) directly after biopsied, snap-frozen in liquid nitrogen and stored at 80°C. Cryostat sectioning was

done to perform serial cryosections (4 μm) on a Leica CM1860 cryostat at −25°C. Cryosections were fixed in cold acetone, washed with PBS, blocked with a protein-block solution (GBI Labs, Bothell, WA) and incubated overnight with rabbit polyclonal antibody against type VII collagen (Acriz, San Diego, CA), rabbit polyclonal antibody against laminin 332 (Abcam, Cambridge, MA), rat monoclonal antibody against perlecan (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal antibody against CTSG (Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit polyclonal antibody against myeloperoxidase (MPO) (Thermo Fisher Scientific, Waltham, MA), respectively. Slides were washed with PBS and incubated with proper fluorescent-tagged secondary antibody (anti-rabbit Alexa Fluor® 488 for type VII collagen, laminin 332, CTSG and MPO, anti-rat Alexa Fluor® 488 for perlecan (Invitrogen, Carlsbad, CA)). Nuclei were stained with DAPI. The sections were mounted on slides using Immu-Mount™ (Thermo Fisher Scientific, Waltham, MA). Fluorescent images were acquired using the Leica TCS SP8 microscope. Fluorescence intensity was quantified by ImageJ software (National Institutes of Health, Bethesda, MD).

Transmission Electron Microscopy (TEM)

BMZ structures were analyzed using TEM. The dorsal skin of hairless mice was cut into 2-3 mm^2 and fixated overnight at 4°C with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M PBS (pH 7.2). The tissues were dehydrated through graded concentrations of ethanol series (50-100%) and then embedded in EPON 812. Ultrathin sections were cut using an ultramicrotome (Reichert Ultracut S, Reichert, Vienna, Austria), stained with saturated uranyl acetate and lead citrate and examined under TEM (JEOL JEM-1400, Tokyo, Japan). Tissue skin structure was examined on the BMZ and several parameters were analyzed. The disruption of lamina densa of each area was measured and counted on the photographs and expressed as the mean number in seven random fields per mouse (seven mice per group). The length of hemidesmosome and the thickness of lamina densa and lamina lucida were quantified by ImageJ software (National Institutes of Health, Bethesda, MD).

Assessment of wrinkle formation

Wrinkle formation on the dorsal area after chronic exposure to UV irradiation was examined using a skin replica assay. Replicas of dorsal skin of hairless mice were made with silicone rubber using SILFLO resin (Silflo Silicone Impression Materials, Potters Bar, UK) and then analyzed by the Skin-Visiometer® SV 650 software (CK Electronic GmbH, Köln, Germany). This instrument assesses a number of wrinkle parameters, including total wrinkle area, number of wrinkles, wrinkle depth and length.

Statistical analysis

Significance was determined using ANOVA. Data are presented as mean ± SD. *P*-values of less than 0.05 were considered statistically significant.

Results

KPA prevents UV-induced decrease of basement membrane components in chronically UV-irradiated mice

Firstly, we elucidated the role of CTSG in UV-induced changes of basement membrane components as one of the characteristic of photoaging. Expressions of basement membrane components, such as type VII collagen, laminin 332, and perlecan, were clearly decreased in UV-irradiated mice compared with non-UV-irradiated control mice. In contrast, the UV-induced decrease of type VII collagen, laminin 332, and perlecan expressions were prevented by KPA treatment, respectively (Figures 14a and 14b). These results suggest that CTSG may be involved in UV-induced decrease of basement membrane components as topical treatment with CTSG inhibitor, KPA, immediately after UV irradiation prevents this phenomenon.

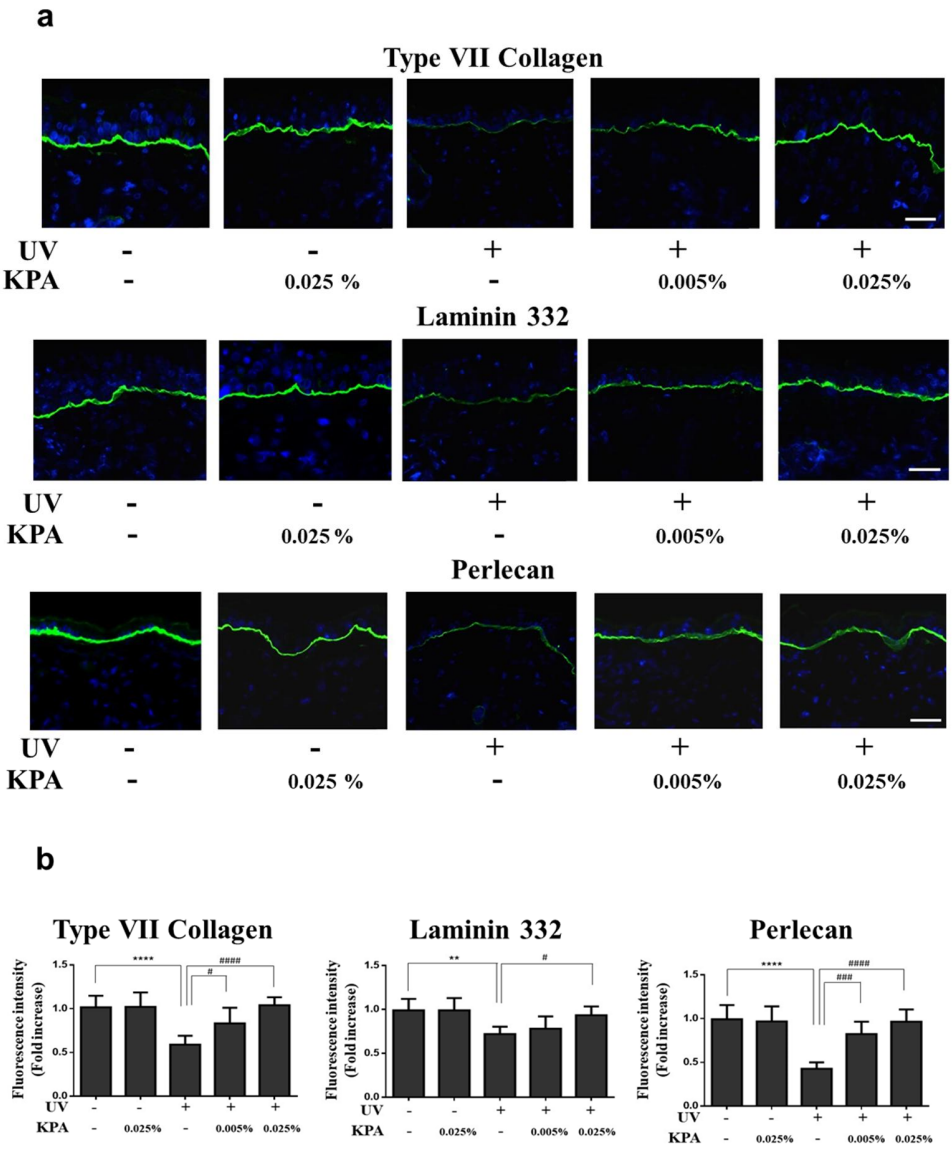

Figure 14. KPA prevents UV-induced decrease of basement membrane components in chronically UV-irradiated mice

The dorsal skin of hairless mice was irradiated with UV 3 times/week for 8 weeks. Sections were subjected to immunofluorescence staining with antibodies against each protein. (a) Representative immunofluorescence staining showing expressions of type VII collagen, laminin 332, and perlecan. Scale bar = 50 μm. (b) Fluorescence intensity was normalized to that of non-UV-irradiated control

mice. Each histogram bar represents the mean value of the normalized and adjusted fluorescence intensity. **P<0.01; ****P<0.0001. $^{\#}$P<0.05, $^{\#\#\#}$P<0.001; $^{\#\#\#\#}$P<0.0001.

KPA prevents UV-induced basement membrane damages in chronically UV-irradiated mice

Chronic exposure to UV is known to cause various histological changes in the skin including damage to the basement membrane structures [5-9]. Since our results indicated that CTSG may be involved in UV-induced decrease of basement membrane components, we investigated whether CTSG is involved in UV-induced basement membrane damages. Ultrastructural analysis revealed that chronic UV irradiation caused breakage/disruption of lamina densa and topical treatment of KPA prevented the UV-induced disruption of lamina densa significantly (Figures 15a and 15b). We also observed that UV increased the thickness of both lamina densa and lamina lucida, in chronically UV-irradiated mouse skin, while KPA inhibited the UV-induced thickening of them. The length of hemidesmosome in chronically UV-irradiated mouse skin was significantly shorter in control mice. Topical application of KPA prevented the shortening of hemidesmosomes by chronic UV exposure (Figures 15a and 15b). Therefore, our results indicate that CTSG may play a crucial role in UV-induced basement membrane damages.

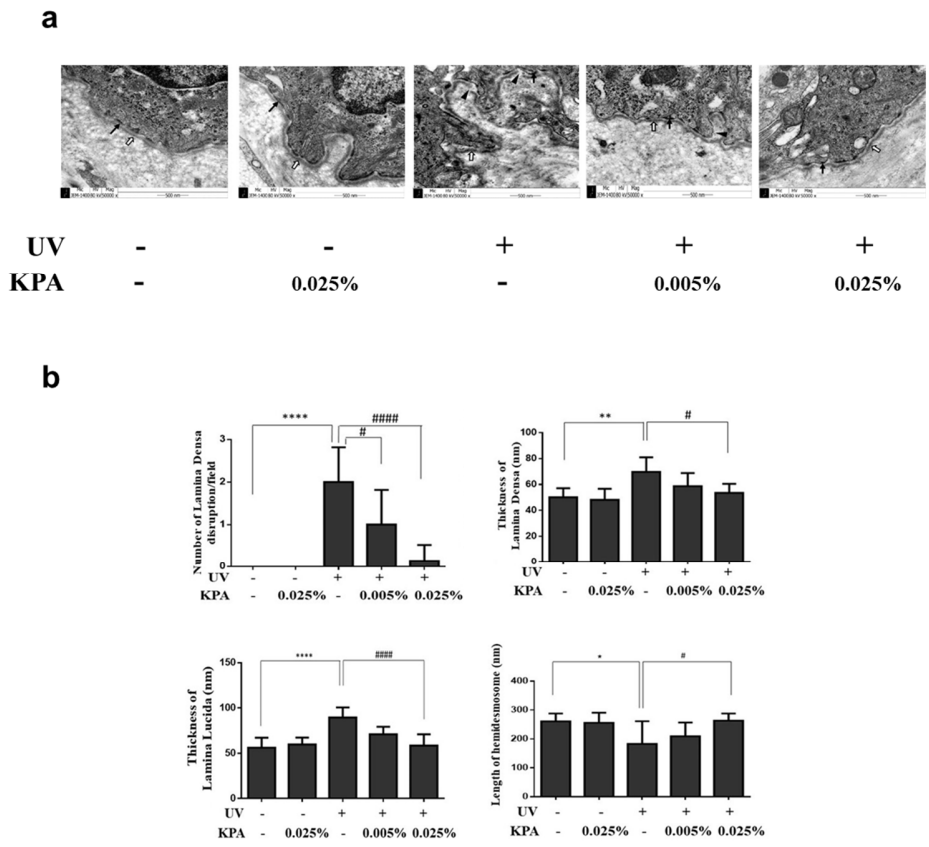

Figure 15. KPA prevents UV-induced basement membrane damages in chronically UV-irradiated mice

Chronic UV irradiation of the dorsal skin of hairless mice was performed as described in the legends of Figure 14. Sections were subjected to TEM analysis. (a) Representative TEM images showing structure of basement membrane in hairless mouse skin. White arrow: lamina densa. Black arrow: hemidesmosome. Arrowhead: disruption of lamina densa. Scale bar = 500 nm. (b) Structure of basement membrane was examined and several parameters were analyzed. *P<0.05; **P<0.01; ****P<0.0001. $^{\#}$P<0.05; $^{\#\#\#\#}$P<0.0001.

KPA prevents UV-induced CTSG expression in chronically UV-irradiated mice

Previous studies have shown that CTSG expression or activity is induced by UV irradiation and is inhibited by KPA treatment in normal human fibroblasts [22, 23]. Therefore, we investigated whether CTSG expression is regulated by UV irradiation in the mouse skin and whether KPA could inhibit UV-induced CTSG expression after chronic UV irradiation. We found that CTSG expression was significantly increased in chronic UV-irradiated mice and it was prevented by KPA treatment (Figures 16a-c). These results suggest that UV-induced CTSG may play an important role in further increase of CTSG in chronically UV-irradiated skin.

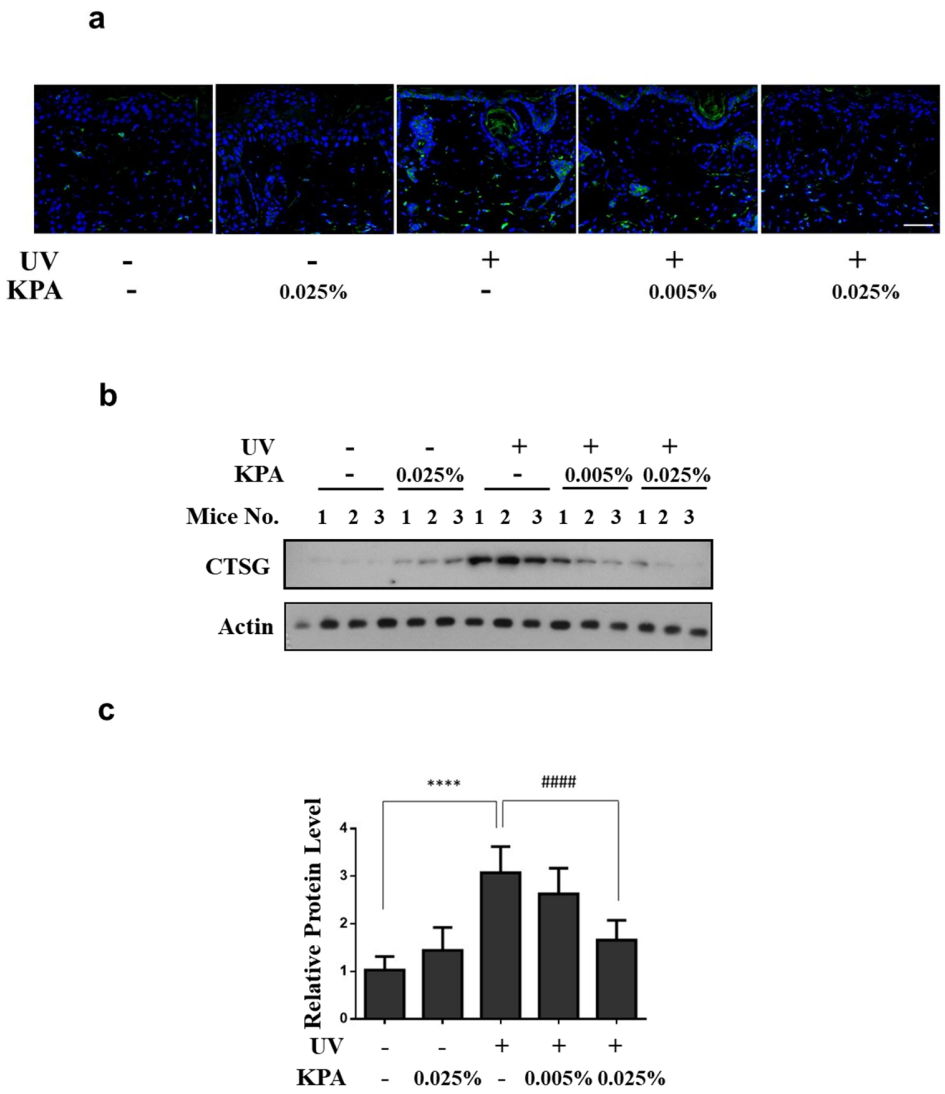

Figure 16. KPA prevents UV-induced CTSG expression in chronically UV-irradiated mice

The dorsal skin of hairless mice was irradiated with UV 3 times/week for 8 weeks. (a) Sections were subjected to immunofluorescence staining with antibody against CTSG. Representative immunofluorescence staining showing CTSG expression in hairless mouse skin. Scale bar = 50 μm. (b) Dorsal skin extracts were subjected to immunoblotting using antibody against CTSG. (c) Relative protein level was quantified by ImageJ software. β-actin was used as a loading control. No. 1-3 represents protein levels of each mouse (seven mice per group). ****P<0.0001; $^{####}$P<0.0001.

KPA prevents UV-induced neutrophil infiltration in chronically UV-irradiated mice

It is well known that acute exposure to UV can increase neutrophil recruitment in mouse and human skin [59, 60]. We investigated whether CTSG could also play a role in UV-induced neutrophil infiltration. We found that neutrophil, expressing myeloperoxidase, infiltration was significantly increased in UV-irradiated mouse skin and that UV-induced neutrophil infiltration was prevented significantly by KPA (0.025%) treatment (Figure 17). This result suggests that UV-induced CTSG may be involved in UV-induced neutrophil infiltration.

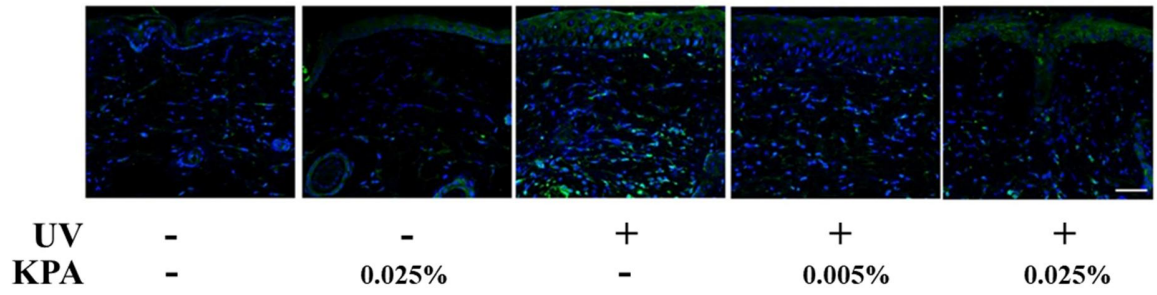

Figure 17. KPA prevents UV-induced neutrophil infiltration in chronically UV-irradiated mice

Chronic UV irradiation of the dorsal skin of hairless mice was repeated 3 times/week for 8 weeks. Sections were subjected to immunofluorescence staining with antibody against MPO. Representative immunofluorescence staining showing expression of neutrophil infiltration in hairless mouse skin. Scale bar = 50 μm.

KPA prevents UV-induced MMP-13 expression in chronically UV-irradiated mice

Next, we investigated whether KPA could inhibit UV-induced MMP-13 expression after chronic UV irradiation. We found that MMP-13 expression was significantly increased in chronic UV-irradiated mice and that KPA treatment inhibited UV-induced MMP-13 expression (Figures 18a and 18b). These results suggest that UV-induced CTSG may play a key role in the increase of MMP-13 expression in chronically UV-irradiated skin.

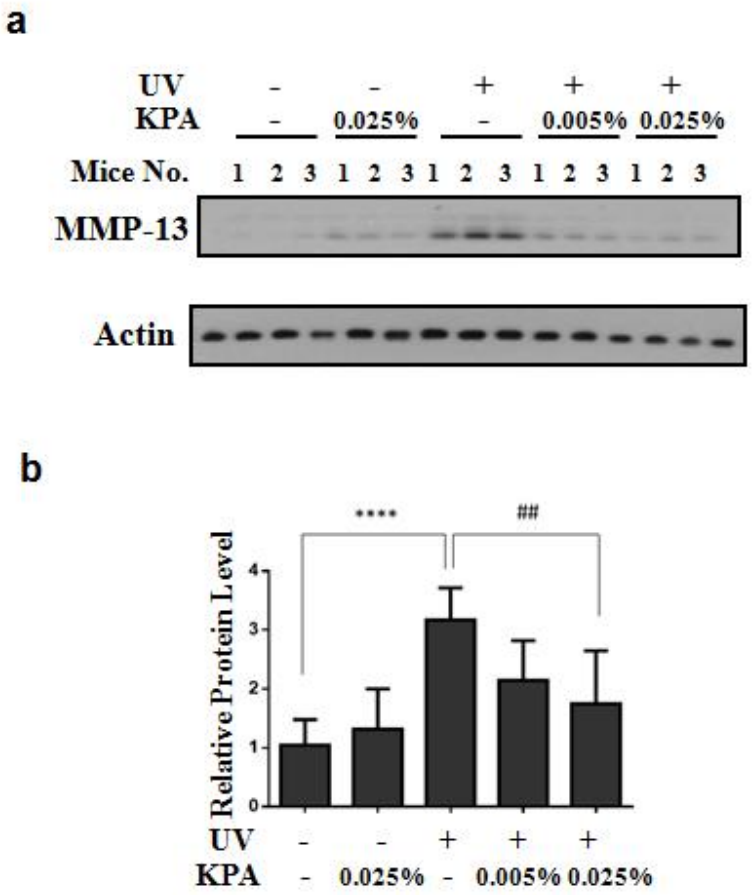

Figure 18. KPA prevents UV-induced MMP-13 expression in chronically UV-irradiated mice

Chronic UV irradiation was performed on the dorsal skin of hairless mice 3 times/week for 8 weeks. (a) Dorsal skin extracts were subjected to immunoblotting using antibody against MMP-13. (b) Relative protein level was quantified by ImageJ software. β-actin was used as a loading control. No. 1-3 represents protein levels of each mouse (seven mice per group). ****P<0.0001; $^{\#\#}$P<0.01.

KPA prevents UV-induced skin wrinkle formation in chronically UV-irradiated mice

In this study, our results showed that CTSG is involved in UV-induced decrease of basement membrane components and the destruction of basement membrane structures. Therefore, we wondered whether these alteration of BMZ may contribute to UV-induced wrinkle formation. We found that topical application of KPA significantly prevented UV-induced wrinkle formation on the dorsal skin of hairless mice (Figures 19a and 19b), suggesting that UV-induced alteration of BMZ may contribute to wrinkle formation in part. Taken together, our results demonstrated that increased CTSG expression may play an important role in UV-induced skin aging and inhibition of CTSG could prevent wrinkle formation in chronically UV-irradiated mouse skin.

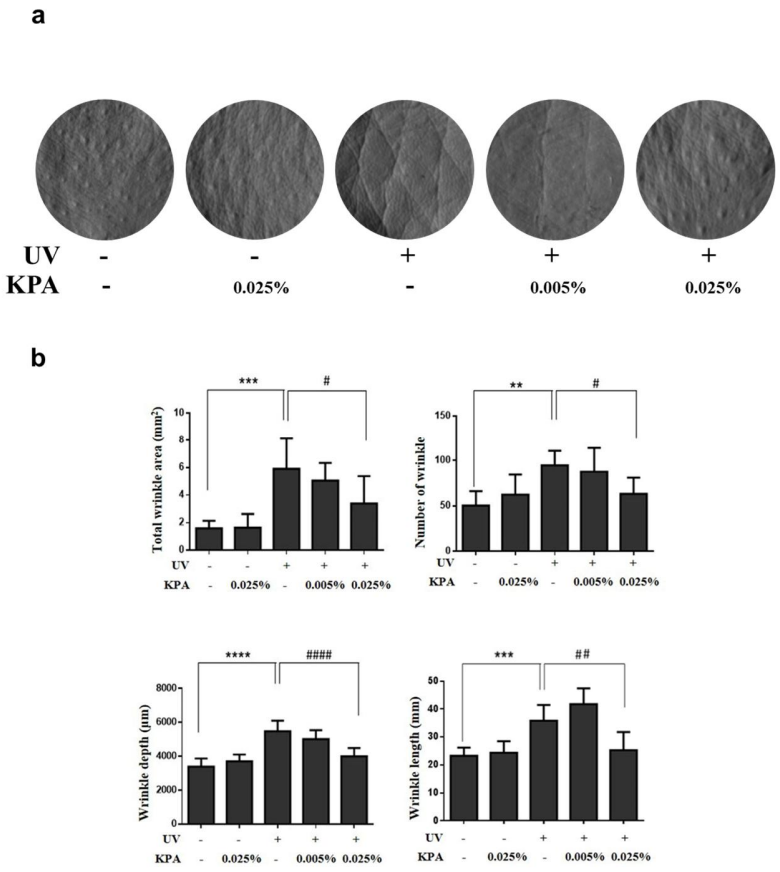

Figure 19. KPA prevents UV-induced skin wrinkle formation in chronically UV-irradiated mice

Chronic UV irradiation was performed on the dorsal skin of hairless mice 3 times/week. (a) Skin replica analysis and (b) graphical representation of the replica image analysis were performed after 8 weeks. **P<0.01; ***P<0.001; ****P<0.0001. $^{\#}$P<0.05; $^{\#\#}$P<0.01; $^{\#\#\#\#}$P<0.0001.

CHAPTER IV

Screening of Cathepsin G Inhibitor using Plant Extracts and Drugs

Materials and Methods

Materials

Cathepsin G (CTSG) inhibitor screening assay kit (colorimetric) was purchased from Abcam (Cambridge, MA) and used following manufacturer's instructions.

Screening compounds

Plant extracts was purchased from BioSpectrum (Yongin, Gyeonggi, Korea). Drugs was obtained from FDA list. Samples were diluted in appropriate vehicle following manufacturer's instructions. Samples and controls were prepared as a set.

Assay procedure

All materials and reagents were prepared to room temperature prior to use. Sample wells contained 10 μl test inhibitors, inhibitor control wells contained 1 μl CTSG inhibitor (0.5 mM) and 9 μl CTSG assay buffer, and enzyme control wells contained 10 μl CTSG assay buffer. CTSG enzyme solution which consisted of CTSG assay buffer (49 μl) and CTSG enzyme (1 μl) was added into each well and incubated at room temperature for 15 minutes. CTSG substrate solution which consisted of CTSG assay buffer (38 μl) and CTSG substrate (2 μl) was added into each well and measured absorbance on a microplate reader at OD 405nm in a kinetic mode, every 3 minutes for 2 hours at 37°C protected from light.

Data analysis

The absorbance values for each sample and control were plotted as a function of the final concentration of compound. The slope for all samples (S), inhibition control (IC) and enzyme control (EC) were calculated as follows

$$\text{Slope} = \left(\frac{A2 - A1}{T2 - T1} \right) \times D$$

A1 = Absorbance value at OD 405 nm at time T1

A2 = Absorbance value at OD 405 nm at time T2

T1 = Time of the first reading (A1) in minutes

T2 = Time of the second reading (A2) in minutes

D = Sample dilution factor

The % relative inhibition was calculated as follows

$$\% \text{ relative inhibition} = \frac{\text{slope of EC - slope of S}}{\text{slope of EC}} \times 100$$

Results

Magnolia liliflora bud extract, Herbex resveratrol, Jeju indicum, and triamcinolone acetonide have high percentage of relative inhibition against CTSG

To discover other substances that are safe for clinical application in human skin instead of β-keto-phosphonic acid (KPA), we performed a screening experiment using plant extracts and drugs from FDA list. The CTSG inhibitor screening assay kit uses the ability of active CTSG to cleave a synthetic pNA (4-Nitroaniline)-based peptide substrate to release pNA. In the presence of a CTSG inhibitor, the cleavage of this substrate is reduced/abolished resulting in decrease or total loss of the pNA absorbance. Our result shows that Magnolia liliflora bud extract and Herbex resveratrol have 80% relative inhibition against CTSG, while Jeju indicum has 90% relative inhibition against CTSG (Table 1). In addition, we show that triamcinolone acetonide has the highest percentage (87.5%) relative inhibition against CTSG among other drugs (Table 2).

Table 1. List of the percentage of relative inhibition of plant extracts against CTSG

Plant extracts	% relative inhibition
승마/Black cohosh	8.33%
Pomegranate	8.33%
Aloe	8.33%
Cashewnut	8.33%
Natural coffee	8.33%
Angelica	8.33%
Iris	8.33%
Made white	20%
Eternal P	20%
Herbex centella extract	20%
Broccoli	25%
Fuligo	40%
Herbex echinacea extract	40%
Jeju nappas	40%
Akebisol	40%
Jeju calamus	40%
Jeju opuntia	40%
Apple stem	40%
Herbex red clover extract	40%
Red snow	40%
Odetox	60%
Jeju lemon	60%

Plant extracts	**% relative inhibition**
Herbex gojiberry extract	60%
MultiEx BSASM plus	60%
엉겅퀴/Sylibum marianum	66.67%
Mulano/Magnolia liliflora	80%
Herbex resveratrol	80%
Jeju indicum	90%

Table 2. List of the percentage of relative inhibition of drugs against CTSG

Drugs	% relative inhibition
Acitretin	25%
Ketoprofen	25%
Celecoxib	25%
Mesalamine	25%
Crotamiton	25%
Diclofenac	25%
Metronidazole	25%
Brimonidine	25%
Ciclopirox	25%
Cysteamine	25%
Eflornithine	25%
Mequinol	25%
Tretinon	25%
Azelastine	37.5%
Ibuprofen	50%
Piroxicam	50%
Desloratadine	50%
Amlexanox	50%
Dapsone	50%
Epinastine	50%
Mafenide	50%
Methoxsalen	50%

Drugs	% relative inhibition
Nepafenac	50%
Niacin	50%
Permethrin	50%
Sulfacetamide	50%
Sulfamethoxazole	50%
Tazarotene	50%
Cromolyn	50%
Doxycycline	50%
Finasteride	50%
Ganciclovir	50%
Sulfasalazine	50%
Fluorometholone	50%
Oxytetracycline	50%
Ketokonazole	62.5%
Dexamethasone	62.5%
Moxifloxacin	62.5%
Meloxicam	62.5%
Calcipotriene	62.5%
Clindanycin	62.5%
Meropenem	62.5%
Chloramphenicol	62.5%
Ciprofloxacin	62.5%
Fluconazole	62.5%
Gatifloxacin	62.5%

Drugs	% relative inhibition
Itraconazole	62.5%
Levofloxacin	62.5%
Norfloxacin	62.5%
Butoconazole	62.5%
Clotrimazole	62.5%
Cyclobenzaprine	62.5%
Dutasteride	62.5%
Halcinonide	62.5%
Isotretinoin	62.5%
Lindane	62.5%
Miconazol	62.5%
Naftifine	62.5%
Silver sulfadiazine	75%
Mycophenolate mofetil	75%
Minocycline	75%
Ofloxacin	75%
Oxiconazole	75%
Penicillin	75%
Bromfenac	75%
Chlorhexidine	75%
Econazole	75%
Fexofenadine	75%
Flunisolide	75%
Griseofulvin	75%

Drugs	% relative inhibition
Ketorolac	75%
Malathion	75%
Terconazole	75%
Triamcinolone acetonide	87.5%

DISCUSSION

Ultraviolet (UV) radiation is a major environmental factor that influences living creatures. The skin is the largest human organ and frequently exposed to UV radiation. Exposure of the skin to UV irradiation is known to induce various skin damages including skin pigmentation, sunburn, inflammation, carcinogenesis, and premature aging, through multiple and complex molecular signaling pathways [7, 61-63]. UV also has been shown to regulate the expressions of many genes [10-12]. However, the roles of Gasdermin C (GSDMC) and Cathepsin G (CTSG) in UV-induced skin damages remain poorly understood.

In our laboratory, studies have been conducted to understand UV-induced skin damage and skin aging at the molecular levels and elucidate how UV regulates the expressions of various MMPs in mouse or human skin in vivo and in skin cells [64, 65]. While investigating the genes whose expressions are changed by UV irradiation in hairless mouse skin using microarray, GSDMC expression was found to be significantly increased by UV irradiation (data not shown). Therefore, in Chapter I, I investigated the role of GSDMC in UV-regulated events in the skin, particularly in UV-induced expressions of matrix metalloproteinases (MMPs) such as MMP-1, MMP-3, and MMP-9 in human skin keratinocytes. These three MMPs are known to play crucial roles in destruction of extracellular proteins by UV radiation [11, 12, 29] and induced by UV radiation at both protein and mRNA levels [12, 66-70]. We first examined whether the expression of GSDMC is regulated by UV radiation. We found that GSDMC expression was decreased at an early time point (8 hours after UV irradiation). A previous study demonstrated that UV-induced specific down-regulation of some gene expressions can occur at early time points. These biological processes related to regulation of transcription, purine nucleotide binding, protein modification, protein amino acid phosphorylation, protein serine/threonine kinase activity, ubiquitin cycle, and cell growth were over-represented by these genes [63, 71, 72]. However, we observed that GSDMC

expression was significantly increased by UV irradiation from 20 hours in human skin keratinocytes (both primary human skin keratinocytes and an immortalized human skin keratinocyte cell line, HaCaT). Then, we studied UV-regulated expressions of GSDMC along with MMP-1, MMP-3, and MMP-9, in more detail, using time-course experiments. Our results indicate that, after UV irradiation, the induction time points of MMP-1, MMP-3 or MMP-9 mRNA expressions were a little faster than those of GSDMC mRNA and protein expressions. At a glance, these results seem to suggest that GSDMC expression may be simply induced by UV radiation, in parallel to or following the expressions of MMP-1, MMP-3, and MMP-9, and, therefore, that this GSDMC induction may have nothing to do with UV-induced MMP expression. However, there are several reports showing that the expressions of certain genes including MMP-1 and type I procollagen can be regulated by multiple factors and pathways. It has been reported that prostaglandin E2 (PGE2) increased by TNF-α treatment at late time points suppresses TNF-α-induced MMP-1 expression in synovial fibroblasts [73] and that TNF-α-induced MMP-1 expressions can be inhibited by the elevation of cAMP or inhibition of GSK-3 activity even after several hours post-TNF-α treatment [42]. Other studies have shown that UV irradiation induces thrombospondin-1 at late time points such as 24 and 48 hours, which attenuates the decrease of type I procollagen expression by UV radiation in human dermal fibroblasts [74]. Collectively, these studies suggest that various factors or signaling pathways, activated after or maintained until several hours after stimulus-treatment, can affect the stimulus-dependent gene expressions. Therefore, we speculated whether the GSDMC increased by UV radiation could affect UV-induced MMP expressions. To test this possibility, we first performed GSDMC expression knockdown, irradiated cells with UV, and then harvested them at 24 and 48 hours. We found that GSDMC expression knockdown decreased the UV-induced expressions of MMP-1 specifically, but not of MMP-3 and MMP-9. These results suggest that, along with other factors or signals regulated by UV at various time points after UV irradiation, the increase in GSDMC expression induced by UV radiation at relatively late time points can contribute to UV-induced MMP-1 expression in human skin keratinocytes.

Many studies have shown that MMP-1 expression can be differentially regulated by various signaling pathways, depending on stimuli and/or cell types [10, 31-33, 39, 42, 47, 75]. However, MAPK signaling pathways such as ERK, JNK, and p38 MAPK are suggested to play central roles in the regulation of MMP-1 expression by diverse stimuli including UV and proinflammatory cytokines [10, 32, 42]. Therefore, we determined whether GSDMC might affect the activations of MAPKs and then examined the regulation of these MAPKs by UV radiation, in more detail, using time-course experiments. Some studies had shown that MAPKs are rapidly activated, probably within 30 minutes after UV irradiation [76, 77]. However, since our results showed that GSDMC expression is increased at relatively late time points after UV irradiation, we decided to investigate the activation of MAPKs by UV radiation from 2 hours to 24 hours, in order to observe whether there is any correlation between GSDMC expression and MAPKs activation. We found that, within the time period used in our experiments, the first activation of ERK and JNK occurred at early time points (2 and 4 hours after UV irradiation) and the second activation occurred at late time points (24 hours after UV irradiation), unlike p38 MAPK activation. Because GSDMC protein levels increased significantly at 20 hours after UV irradiation, we speculated whether GSDMC might affect the second activation of ERK and JNK. Therefore, we first performed GSDMC expression knockdown and then assessed activations of ERK, JNK and p38 MAPK induced by UV irradiation. We found that the activations of ERK and JNK induced by UV irradiation, at late time points but not at early time points, were significantly reduced by GSDMC expression knockdown. However, UV-induced activation of p38 MAPK was not affected by GSDMC expression knockdown. These results indicate that the induction of GSDMC by UV radiation at relatively late time points plays an important role in controlling UV-induced ERK and JNK activations. Even though GSDMC may function as an oncogene and AP-1 has been known to play an important role in cell proliferation [17, 18, 78, 79], cell growth in our experiment was normal. This might be due to serum-starved condition as pretreatment before cell transfection and post-treatment after cell transfection. Next, to confirm the results obtained from our GSDMC knockdown experiments, we overexpressed

GSDMC and checked MMPs expressions and MAPKs activations. We found that overexpression of GSDMC increased the expression of MMP-1 but not of MMP-3 and MMP-9 and induced the activation of ERK and JNK but not of p38 MAPK. Furthermore, we found that GSDMC-induced MMP-1 expression was suppressed by treatment with ERK or JNK inhibitors. Therefore, these results establish that GSDMC can regulateMMP-1 expression via activation of ERK and JNK pathways. Taken together, our results indicate that the induction of GSDMC expression by UV radiation increases the activities of ERK and JNK, which leads to further increasing MMP-1 levels at the late time points such as 24 and 48 hours after UV irradiation.

The ERK and JNK pathways are well known to play critical roles in UV-induced MMP-1 expression [10, 11]. However, how the activations of ERK and JNK pathways are regulated by UV radiation is not completely understood. Our present results with the published studies show that the activations of ERK and JNK pathways can occur at not only relatively early time points but also relatively late time points after UV irradiation in human skin keratinocytes. The activations of ERK and JNK pathways at early time points after UV irradiation are due to activation of cell surface receptors such as EGFR [43, 44], whereas the activations of ERK and JNK pathways at late time points after UV irradiation are due to increase in GSDMC expression. In addition, the results showing that GSDMC expression knockdown reduces the expression of MMP-1 and activations of ERK and JNK induced by UV radiation suggest that the late activations of ERK and JNK pathways play significant roles in UV-induced MMP-1 expression. Thus, the activations of ERK and JNK pathways at both early and late time points may contribute to full induction of MMP-1 expression by UV irradiation in human skin keratinocytes. Therefore, our results help us to better understand UV-induced activations of ERK and JNK pathways and MMP-1 expression in a time-dependent manner and the role of GSDMC in the process.

In addition to elucidating the role of GSDMC in UV-induced MMP-1 expression, our present work suggests that the expressions of MMP-1, MMP-3, or MMP-9 may be distinctively regulated by ERK and JNK pathways. These results may seem to be somewhat contradictory to the published

reports showing that ERK or JNK activities play critical roles in the regulation of MMP-3 or MMP-9 expressions [67-69, 80]. However, unlike to the experiments used in our study, in most studies, the ERK or the JNK pathways were inhibited from very early time by treating with specific inhibitors before or immediately after stimuli-treatment [68, 69, 80]. The activities of ERK and JNK may be very important for the expressions of all of MMP-1, MMP-3, and MMP-9. However, when their activities are required for efficient and full expression can be different among MMP-1, MMP-3, and MMP-9. The activations of ERK and JNK pathways at early time points such as 2 or 4 hours after UV treatment may be critical for expression of all MMP-1, MMP-3, and MMP-9, while the activations of ERK and JNK pathways at late time points such as 20 or 24 hours after UV treatment may be important for further induction of MMP-1 but not of MMP-3 and MMP-9.

In Chapter II, I identified the signaling pathways that may be involved in UV-induced GSDMC expression in HaCaT immortalized human keratinocyte cell line and in primary human skin keratinocytes. In our laboratory, TRPV1 and GSDMC were found to play important roles in UV-induced MMP-1 expression in human skin keratinocytes; however, TRPV1 activation occurs at relatively early time points, whereas GSDMC expression increases at relatively late time points, following UV irradiation [39, 46]. Therefore, it was hypothesized that TRPV1 may play a role in UV-induced GSDMC expression. The results indicated that inhibition of TRPV1 activity suppressed UV-induced GSDMC expression. In addition, direct activation of TRPV1 by capsaicin increases GSDMC expression. These results indicated that TRPV1 may play an important role in GSDMC expression. It has been reported that TRPV1 acts as a non-selective cation channel and that the activation of TRPV1 may lead to calcium influx [39, 47, 51]. As the results from the present study indicated that TRPV1 may be involved in UV-induced GSDMC expression, the role of extracellular calcium on UV-induced GSDMC expression was examined and it was demonstrated that UV-induced GSDMC expression was calcium-dependent. Calcium is known to modulate several proteins, such as calcium-binding protein calmodulin, kinases and phosphatases [54, 81, 82]. Previous studies reported that calcineurin is a calcium and calmodulin-dependent serine/threonine

protein phosphatase [52, 53]. The present study results demonstrated that UV-induced GSDMC expression was calcium-dependent; therefore, whether calcineurin may be involved in UV-induced GSDMC expression was investigated. The data revealed that the calcineurin pathway may play an important role in UV-induced GSDMC expression.

Calcineurin is known to activate NFATc family members by dephosphorylating them [54-57]. A previous study demonstrated that UV is a strong inducer for NFATc1 transactivation and that UV induces NFATc1 by activating calcium/calcineurin pathway in skin [83]. UV induces transcriptional activity and nuclear translocation of NFATc1 in human skin keratinocytes [84]. Furthermore, UV is known to enhance NFATc1 binding activity to DNA. NFATc1 binds DNA cooperatively with other transcription factors to increase transcription of certain genes [85]. These results indicated that NFATc1 may be activated by UV in human skin keratinocytes. Therefore, the present study aimed to determine whether NFATc1 was involved in UV-induced GSDMC expression. The results demonstrated that UV-induced GSDMC expression may be mediated through NFATc1. Taken together, the present study results indicated that the TRPV1/calcium/calcineurin/NFATc1 signaling pathway may be involved in UV-induced GSDMC expression in human skin keratinocytes.

The present study findings may help us to not only identify the molecular mechanisms involved in UV-induced GSDMC expression, but also to better understand the signaling pathways involved in UV-induced MMP-1 expression. The induction of MMP-1 expression by UV may be regulated by various factors and signaling pathways. The activation of cell surface receptors including EGFR by UV induces signal transduction cascades and activates various signaling pathways, such as ERK and JNK, that are known to be important for MMP-1 expression [43, 44]. In addition, TRPV1 may also be activated by UV and plays a crucial role in UV-induced MMP-1 expression [39, 47]. Even though the mechanism through which TRPV1 is activated by UV remains unclear, it has been reported that Src kinase mediates UV-induced TRPV1 trafficking from a vesicle inside cytoplasm to cell membrane within 15 minutes following UV irradiation, which was

suggested to be an important step in TRPV1 activation [41]. The activation of TRPV1 at relatively early time points by UV turns on the calcium/calcineurin/NFATc1 signaling pathway, which finally increases GSDMC expression at relatively late time points after UV irradiation. The increase of GSDMC expression activates ERK and JNK pathways, leading to the induction of MMP-1 expression.

In addition, the involvement of the EGFR pathway in UV-induced GSDMC expression in HaCaT cells was also examined in the present study; however, the inhibition of EGFR by the chemical EGFR inhibitor (AG1478) did not affect UV-mediated GSDMC induction (data not shown). These results indicated that UV-induced GSDMC expression may occur independent of the EGFR pathway, and may be through the TRPV1 pathway. However, it has been reported that EGFR plays a critical role in UV-induced MMP-1 expression [11, 26, 86]. Therefore, these previous and present results further support our hypothesis, which suggested that several signaling pathways are involved in UV-induced MMP-1 expression.

In Chapter III, I showed that KPA, the strongest known CTSG inhibitor, prevents UV-induced decrease of basement membrane components, damage of basement membrane ultrastructure, UV-induced increase of CTSG and MMP-13 expressions, neutrophil infiltration in chronically UV-irradiated hairless mice. Furthermore, UV-induced wrinkle formation was also prevented by topical treatment of KPA.

The basement membrane zone (BMZ) is a complex structure that anchors the overlying epidermis to the dermis [87, 88]. The BMZ plays a crucial role in maintaining integrity of the skin and in regulating various important processes such as cell differentiation, formation of permeability barriers, and extracellular signals [88, 89]. The major components of the BMZ are laminin, type VII collagen, heparan sulfate proteoglycans such as perlecan, and type IV collagen. These proteins are important in constructing the primary scaffolding structure of the BMZ [88]. Laminin is one of the most abundant non-collagenous glycoproteins of the BMZ. Laminin 332 is the main constituent of the anchoring complex, which interacts with type VII collagen, to assemble the anchoring fibrils.

Laminin 332 can also bind to perlecan via α chain G4 domains, to form an electron-dense zone called lamina densa [88, 90-92]. Laminin, collagens, and proteoglycans are also known to be cleaved by cathepsin G (CTSG) in bovine cartilage matrix and basement membrane of mouse kidney [93-95]. Previous study reported that a serine protease inhibitor, which inhibits CTSG, binds to laminin 332, type VII collagen, and heparan sulfate, but not to laminin 111, type IV collagen, and fibronectin [96]. In this study, we found that the type VII collagen, laminin 332, and perlecan expressions in the BMZ, were significantly decreased in UV-irradiated mice compared with non-UV-irradiated control mice. And we demonstrated that UV-induced decreases of these three proteins were markedly prevented by topical application of KPA immediately after UV irradiation. These results indicate that CTSG may play an important role in reduction of basement membrane components in chronically UV-irradiated hairless mice.

Since CTSG may be involved in UV-induced decreased expression of basement membrane components, we investigated whether increase of CTSG by UV might also have effect on UV-induced destruction of basement membrane structures. Electron microscopic observation revealed that the ultrastructure of BMZ were significantly altered by chronic UV exposure, which were characterized by severe disruption of lamina densa, the increased thickness of lamina densa, and the decreased length of hemidesmosome. We also observed that the lamina lucida thickness was significantly increased by chronic UV irradiation. In contrast, the structure of basement membrane in UV-irradiated and KPA (0.025%)-treated mice was nearly similar with those in non-UV-irradiated control mice. Taken together, our results suggest that CTSG may play a crucial role in UV-induced destruction of basement membrane structures.

The expression or activity of CTSG has been known to be induced by UV irradiation and inhibited by KPA treatment in normal human fibroblasts [22, 23]. Consistent with previous studies, we also found that CTSG expression was induced by chronic UV irradiation and topical KPA treatment inhibited UV-induced CTSG expression. Since CTSG is shown to be expressed in leukocytes [19-21], we decided to confirm neutrophil recruitment by immunofluorescence staining.

We found that UV induced neutrophil infiltration and topical KPA treatment inhibited UV-induced neutrophil infiltration. There are several studies showing that CTSG inhibitor is capable of reducing influx of neutrophil into the inflammation sites significantly [97-99]. Taken together, these results may indicate that the expression of CTSG may be related to the neutrophil infiltration.

It is well known that various MMPs expressions are induced by chronic UV irradiation, including MMP-1. Some published works reported that CTSG upregulates the expression of MMP-1 [23, 24]. Since MMP-1 is functionally replaced by MMP-13 in rodents [34-37], then we observed the expression of MMP-13 in chronically UV-irradiated hairless mouse skin. We found that MMP-13 expression was significantly increased in chronic UV-irradiated mice and topical KPA treatment prevented it. Previous study showed that CTSG induces the cleavage of phospholipid transfer protein (PLTP) and that the loss activity of PLTP led to the increased expression of MMP-13 [99]. Therefore, our results suggest that UV-induced CTSG may play an important role in the increase of MMP-13 expression in chronically UV-irradiated mice.

Repeated UV exposure over a prolonged period induces the expression of CTSG that leads to degradation of basement membrane components [23, 24]. Therefore, we wanted to investigate whether CTSG-mediated basement membrane damages may contribute to the UV-induced wrinkle formation. We demonstrated that UV increases the total wrinkle area, number of wrinkles, wrinkle depth and length in UV-irradiated mice, but concomitant topical treatment with KPA prevents wrinkle formation. Other previous studies have suggested that CTSG may contribute to wrinkle formation through fibronectin fragmentation and MMP activation [23, 24]. Possibly, UV-induced wrinkle formation can occur not only by MMPs-mediated ECM degradation, but also by CTSG-mediated basement membrane damages. Therefore, we suggest that UV-induced CTSG-mediated damage of basement membrane may contribute to the wrinkle formation in part.

In the last chapter, Chapter IV, I performed a screening experiment using plant extracts and drugs from FDA list to determine other substances that are safe for clinical application in human skin instead of KPA. Our result shows that, among other plant extracts, Magnolia liliflora bud

extract and Herbex resveratrol have 80% relative inhibition against CTSG, while Jeju indicum has 90% relative inhibition against CTSG. In addition, among other drugs, triamcinolone acetonide has the highest percentage (87.5%) relative inhibition against CTSG.

Magnolia liliflora is reported to have antiinflammatory, antimicrobial and antiallergic activities. It is a commonly used Chinese medicinal herb for symptomatic relief of allergic rhinitis, sinusitis and headache. It has been shown to inhibit various allergic and inflammatory responses induced by mediators, such as histamine, prostaglandin E_2 (PGE_2), platelet active factor (PAF) and nitric oxide (NO), pro-inflammatory cytokines interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) [100]. Previous study suggests that Magnolia liliflora inhibits release of mast cell-derived histamine in rat peritoneal mast cells [101]. Another study shows that Magnolia liliflora has a potential role as a natural antioxidant and antidermatophytic agent [102].

Resveratrol is known to possess cardio-protective, antioxidant, neuroprotective, immunomodulatory, anti-inflammatory, metabolism-regulating, and anti-cancer effects [103, 104]. An accumulating body of evidence has shown that resveratrol inhibits cell growth and induces apoptosis in various melanoma cells by S-phase cell cycle arrest and down-regulation of cyclins [105-107]. In addition, Fang *et al.* recently found that resveratrol enhances the sensitivity of melanoma cells to radiation by inhibiting proliferation and inducing apoptosis. The anti-proliferative effects of resveratrol following radiation were associated with decreased expression of the proliferative molecules [108].

Jeju indicum or Rhododendron indicum flower extract may act as an anti-wrinkle. It promotes collagen synthesis of the skin. It is also effective in the treatment of alopecia [109].

Unlike plant extracts, which have been proven safe to be used as cosmetic ingredients, the side effects of drugs used in the long-term clinical application in human skin should be considered. Since triamcinolone acetonide is a synthetic corticosteroid, which has strength potency, it cannot be used in the human skin for a long period [110, 111]. Long-term use of topical steroids is most concerning, and side effects can be either local or systemic. This is worsened by factors such as

higher-potency steroids, occlusion, thinner skin, and older patient age. Local effects may include stinging or burning sensation, skin thinning, stretch marks, rosacea, increased hair growth where the skin is being treated, and allergy. In some cases, the topical steroid penetrates the skin and enters the bloodstream, which is primarily a concern in children who are on high doses as this can affect their growth. Other systemic effects are fluid build-up in the legs, increase in blood pressure, Cushing's syndrome, and bone damage [111, 112].

Instead of triamcinolone acetonide, there are some drugs which have 75% relative inhibition against CTSG. However, among these drugs, minocycline may be preferred for clinical application in the human skin. Minocycline is a broad-spectrum antibiotic that is usually used to treat acne vulgaris. Minocycline bind to the 30S ribosomal subunit, preventing the binding of tRNA to the mRNA-ribosome complex and interfering with protein synthesis. A recent Phase 2b clinical trial evaluated the novel topical gel formulation of the antibiotic minocycline (BPX-01) in patients with moderate-to-severe acne. The results show that BPX-01 is an investigational delivery system that formulates the leading acne antibiotic, minocycline, into a unique, hydrophilic topical gel. The study demonstrates that 1% and 2% doses of BPX-01 significantly reduced the number of inflammatory acne lesions when compared to a vehicle after 12 weeks of treatment [113]. Previous in vitro and in vivo studies demonstrated that a low-dose, topical 1% minocycline gel (BPX-01) provided a localized and targeted delivery of minocycline to the epidermis and pilosebaceous units at efficacious levels. This potentially limits systemic exposure and may reduce treatment side effects. Administration of BPX-01 at 2.5 mg minocycline/cm^2/day was well tolerated, with no significant local or systemic toxic effects. Topical BPX-01 minocycline was only detected in the skin but not in the plasma. BPX-01 is a novel topical gel formulation of minocycline that can be delivered to target areas of acne without side effects and with a favorable safety and efficacy profile [114].

In conclusion, my results in Chapter I explained the role of GSDMC in UV-induced MMP-1 expressions, specifically via activation of ERK and JNK pathways. Since MMP-1 has been known

to be the major collagenolytic enzyme responsible for collagen damage in UV-irradiated human skin; therefore, GSDMC plays an important role in the UV-induced skin damages. To advance the development of strategies and reagents for the prevention or treatment of skin damages, it is important to understand UV-induced skin damages at the molecular level and elucidate how UV radiation regulates GSDMC expression. My findings in Chapter II elucidate the signaling pathway involved in UV-induced GSDMC expression. It shows that TRPV1 may play an important role in the induction of GSDMC expression by UV and that UV-induced GSDMC expression may be mediated via a calcium/calcineurin/NFATc1 pathway. My studies in Chapter III show the involvement of CTSG in UV-induced basement membrane damages in the skin and that inhibition of CTSG may be a useful strategy for the prevention of UV-induced basement membrane damages and photoaging. My screening results in Chapter IV provide some plant extracts and drug that are safe for clinical application in the human skin instead of KPA.

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국문 초록

Gasdermin C (GSDMC)는 Gasdermin family 유전자의 구성원으로 알려져 있으며 피부를 비롯한 다양한 조직의 상피 세포에서 발현된다고 알려져 있다. Gasdermin family members 는 고도의 tissue-specific 의 방식으로 다양한 조직 유형의 상피에서 검출되는 것으로 밝혀졌으며, 분화에 따라 특이적 역할을 할 것으로 예상되고 있다. 그러나 GSDMC 의 피부에서의 기능은 아직 잘 알려져 있지 않다.

Cathepsin G (CTSG)는 serine protease family 에 속하며 마우스 피부에서 만성 자외선 조사에 의한 주름 형성에 관여한다고 알려져 있다. β-keto-phosphonic acid (KPA)는 CTSG 에 대한 강력한 선택성과 효능을 나타내는 CTSG 억제제로 알려져 있다. CTSG 억제제인 KPA 가 만성 자외선 조사에 의한 주름 형성을 예방하는 하는 효과는 잘 알려져 있으나, CTSG 억제제가 피부의 기저막 손상에 미치는 영향에 대해서는 분명하지 않다.

자외선 (UV)은 염증, 일광 화상, 피부 노화, 발암 등 다양한 피부 손상에 중요한 역할을 하며 피부의 기저막 손상도 유발한다. UV 의 이러한 효과는 세포주기 조절, 세포 사멸, 신호 전달 및 유전자 발현과 같은 분자 또는 세포 수준에서의 많은 변화에 의해 조절된다. 그러나 GSDMC 와 CTSG 의 UV 에 의해 유도 된 피부 손상에 관한 역할은 충분히 보고 되지 않고 있다.

제 1 장에서는, 사람 피부 각질형성세포에서 UV 에 의한 MMP-1, MMP-3 및 MMP-9 발현에서 GSDMC 의 역할에 대해 발견하였고 GSDMC 발현이 사람 피부 각질형성세포에서 UV 조사에 의해 증가한다는 것을 관찰하였다. GSDMC 발현은 UV 조사 후 상대적으로 늦은 시점에서 증가하는 것으로 보였고, 이러한 GSDMC 유도는 MMP-3, MMP-9 가 아닌 MMP-1 의 발현 및 ERK 와 JNK 활성화에 중요한 역할을 하였다. 또한, GSDMC 의 과발현은 MMP-1 발현 및 ERK 와 JNK 활성을 증가시키고 GSDMC 에 유도 된 MMP-1 발현은 ERK 또는 JNK 활성의 억제에 의해 억제됨을 발견하였다.

제 2 장에서는, UV 에 의해 유도 된 GSDMC 발현에서 TRPV1 의 역할과 사람 피부 각질형성세포에 관여하는 신호 전달 경로를 관찰하였다. TRPV1 활성의 억제가 UV 에 의해 유도 된 GSDMC 발현을 현저하게 감소시키고 TRPV1 활성화가 GSDMC 발현을 증가시킨다는 것을 발견하였다. 또한, 세포 외 calcium 과 calcineurin 활성이 UV 에 의해 유도 된 GSDMC 발현에 필요하다는 것을 발견하였을 뿐만 아니라 UV 에 의해 유도 된 GSDMC 발현이 NFATc1 의 넉다운 및 과발현에 의해 각각 감소되고 증가하였다.

제 3 장에서는, 만성 UV 조사한 hairless 마우스의 피부에서 기저막 손상에 대한 CTSG 의 역할을 관찰하였다. 본 연구에서는 KPA 가 기저막 (BMZ)에서 자외선에 의한 type VII collagen, laminin 332 및 perlecan 과 같은 기저막 구성 요소의 감소와 UV 에 의한 lamina densa 의 파손과 hemidesmosome 의 축소를 방지하는 것을 관찰하였다. KPA 는 만성 자외선을

조사한 hairless 마우스에서 UV 에 의해 유도 된 CTSG 및 MMP-13 발현을 억제하였다. UV 조사에 의한 호중구 침윤의 증가는 KPA 치료에 의해 억제되었으며, UV 에 의해 형성 된 주름도 국소 KPA 치료로 예방됨을 관찰하였다.

제 4 장에서는 식물 추출물과 약물로 CSTG 억제제의 스크리닝 하여 KPA 대신 사람 피부에 임상응용에 안전한 다른 물질을 찾게 되었다. 다른 식물 추출물과 약물 중에서 각각 제주의 인삼 및 triamcinolone acetonide 가 CTSG 에 대한 억제 활성이 가장 높게 관찰되었다. 그러나, 사람 피부 임상응용에, 특히 장기적인 응용에 사용 할 약물은 부작용을 고려해야 한다.

요약하면, 본 연구에서는 UV 조사에 의한 GSDMC 의 증가가 ERK 와 JNK 경로 활성화를 통하여 UV 에 의해 유도 된 MMP-1 발현에 관여함을 밝혔다. TRPV1 은 UV 유도에 의한 GSDMC 발현에 중요한 역할을 하며 UV 의한 GSDMC 발현은 calcium/calcineurin/NFATc1 경로를 통하여 조절된다는 사실을 규명하였다. 또한 피부에서 UV 에 의한 기저막 손상에 CTSG 가 관여한다고 보여 주었고, CTSG 의 억제는 UV 에 의한 기저막 손상과 광노화를 막을 수 있는 유용한 전략이 될 수 있을 것이다. 본 연구에서는 KPA 대신 인간의 피부에서 CTSG 활성을 억제 할 수 있고 임상적으로 비교적 안전하게 사용 할 수 있는 물질을 발견하였다.